A DISSERTATION
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Nutritional studies on taurine essentiality and requirement in parrot fish (*Oplegnathus fasciatus*) diet for fish meal replacement

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Essentiality and requirement of taurine in diets for parrot fish, (*Oplegnathus fasciatus*)

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Contents

요약문

양어사료에서 주 단백질원으로 사용되는 어분의 대체는 양식산업의 비약적 발전과 어획량 감소로 인해 양식산업에서 중요한 사안으로 부각되고 있다. 식물성 단백질원들은 높은 단백질 함량과 비교적 우수한 아미노산조성으로 어분을 대체할 수 있는 단백질 사료원으로서 양어사료에 이용되고 있다. 양어사료 내 식물성 단백질원의 이용성을 높이기 위한 대부분의 연구는 식물성 단백질원에 함유된 항영양물질을 줄이는데 초점을 두어 수행되고 있으나 일반적으로 육식성 해산어류 사료에 있어 매우 제한적으로 이용된다.

타우린은 육식성 어류가 자연에서 섭취하는 먹이에 다량 함유된 아미노산으로서 양어사료에 있어 비필수아미노산으로 여겨져 왔다. 타우린은 어류의 조직 내 고농도로 존재하며 양어사료에서 타우린의 주요한 공급원은 어분이다. 따라서 식물성 단백질원을 이용한 어분대체는 양어사료 내 타우린의 결핍을 야기 시킬 수 있으며 반대로 식물성 단백질원이 다량 함유된 양어사료 내 타우린의 보충은 식물성 단백질원의 이용을 증대 시킬 수 있을 것이다. 이러한 가설에 착안하여 본 연구는 육식성 해산양식 어류인 돌돔을 대상으로 사료 내 타우린의 첨가가 식물성 단백원의 이용성을 증가 시킬 수 있는지를 타진하고, 더 나아가 돌돔 사료 내 타우린의 적정 요구량 및 필수성을 입증하기 위해 수행됐다.

위에서 언급한 가설을 입증하기 위해 4번의 사양실험을 수행하였으며,

사양실험1은 (Chapter two) 치어기 및 성장기 돌돔 배합사료 내 식물성 단백질원의 이용성을 연구하였으며, 사양실험2와 3은 (Chapter three and four) 식물성 단백질원이 다량 함유된 돌돔 사료 내 타우린의 이용성 및 적정 첨가 함량을 규명하였으며, 사양실험4는 (Chapter five) 돌돔 배합사료 내 타우린의 요구량 및 필수성을 규명하였다.

사양실험1의 결과, 치어기 돌돔 배합사료 내 식물성 단백질원인 면실박과 대두박으로 어분 단백질을 20%까지 대체할 수 있었으나, 30% 이상의 어분대체 실험구에서는 유의적으로 낮은 성장률과 사료효율이 관찰되었다. 이를 바탕으로 사양실험2에서는 대두박을 이용하여 30% 어분단백질을 대체하여 단계적으로 높은 함량의 타우린(0.05%, 1.0%, 2.0%)을 보충하여 실험디자인을 하였다. 그 결과, 치어기 돌돔에 있어서 사료 내 1% 이상의 타우린 첨가 시 어분단백질을 30%까지 대체할 수 있었으며, 타우린 첨가그룹은 무첨가 그룹에 비해 유의적으로 높은 성장률과 사료효율을 나타내었다. 따라서 사양실험3은 더 높은 함량의 식물성 단백질원의 첨가에 따른 타우린의 이용성을 확인하기 위해 대두박을 이용하여 20%, 30%, 40%의 어분단백질을 대체하여 1% 타우린의 각각 보충하였다. 그 결과, 어분대체 비율이 증가 할수록 어류의 성장률과 사료효율은 떨어졌으나, 같은 함량의 어분대체그룹에 있어서 타우린 첨가 그룹들은 모두 유의적으로 높은 성장률과 사료효율을 나타내었다. 대두박 단백질을 이용하여 어분단백질을 40% 대체한 실험구는 유의적으로 가장 낮은 성장률과 사료효율을 나타내었으나, 40% 대체실험구에 타우린을 첨가한 실험구는 대조구와 유의적인 차이를 나타내지 않았다. 이러한 결과들을 바탕으로 하여 사양실험4는 타우린의 요구량 및 필수성을

규명하기 위해 어분이 50% 함유된 돌돔 배합사료 내 단계적으로 높은 함량 (0%, 0.2%, 0.4%, 0.8%, 1.2%, 1.6%)의 타우린을 첨가하였다. 그 결과, 0.8% 이상 타우린이 첨가된 그룹들에서 유의적으로 높은 성장률과 사료효율이 나타났으며, Broken-line regression 분석방법을 이용하여 분석한 결과 성장기 돌돔 배합사료 내 최적의 타우린 요구량은 0.91%로 나타났다.

위 결과들을 종합해 볼 때, 치어기 및 성장기 돌돔 배합사료 내 타우린의 첨가는 식물성 단백질원의 이용성을 증가 시킬 수 있을 것으로 여겨지며, 어분을 기초로 한 사료에 있어서도 타우린의 첨가는 돌돔의 성장 및 사료효율을 증가 시킬 수 있을 것으로 여겨진다. 치어기 및 성장기 돌돔 배합사료 내 타우린의 적정 요구량은 1% 내외로 판단되며, 이러한 결과들을 바탕으로 하였을 때 타우린은 돌돔 배합사료 내 최적의 성장을 위한 필수아미노산으로 여겨진다.

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CHAPTER ONE: Literature review

1.1. Aquaculture trends

Over the last three decades the aquaculture industry has exhibited the fastest growth rates of any food producing business with an average of 8.8% per year, which is approximately seven and three times higher than growth rates observed for capture fisheries and terrestrial farm meat production, respectively (FAO, 2006). Aquaculture contributed 43 percent of aquatic animal food for human consumption in 2007 (e.g. fish, crustaceans and molluscs) and is expected to grow further to meet the future demand. The rapid growth in the production of carnivorous species such as salmon and marine fish has been driven by globalizing trade and favorable economics of larger scale intensive farming. Projections by FAO suggest that by 2030, aquaculture production will need to be more than double compared to production levels of 2006 (FAO, 2008).

The expansion of aquaculture production has been accompanied by rapid growth of aquafeed production. The facing challenge in the current aquaculture industry is to identify economically viable and environment-friendly alternatives to fish meal (FM) and fish oil on which many present aquafeeds are largely based. While the supply of FM and oil is arguably sustainable, the anticipated growth in demand internationally for aquafeeds is presumed to exceed the supply in the next decade. Thus, the aquafeed industry has recognized for many years that viable utilization of plant feedstuffs for the production of cold and warm water aquatic species is one of the most urgent requirements for the future aquaculture development.

The plant feedstuffs must provide nutritious diets that will effectively grow aquatic species with minimal environmental impact and produce high-quality fish flesh to confer human health benefits in a cost-effective manner. As the aquaculture industry continues to expand on a global scale, access to key feedstuffs, such as FM and oil, will become increasingly limited because of a finite wild-harvest resource. In addition to concerns about the sustainability of fisheries resources, other issues including the potential presence of organic and inorganic contaminants in FM and the net effect of demand-and-supply economics in the global market require enhanced efforts to thoroughly evaluate those plant feedstuffs.

1.2. Replacing fish meal as plant protein sources

FM is a principal protein source in carnivorous fish diets and is responsible for the major proportion of diet cost. For that reason and also because of the industries' need for flexibility in choice of ingredients in a constantly changing raw material market, reduction in FM inclusion level in practical aquafeeds is a priority (Tacon, 2000). Moreover, the growth in aquaculture industry, not followed by growth in wild catch, has enforced a shift in the use of protein sources from FM toward sustainable plant feedstuffs (Rumsey et al., 1993; Gomes et al., 1995).

The dietary use of plant species like barley (Cheng and Hardy, 2002; Overturf et al., 2003), cottonseed meal (Lim and Lee, 2008, 2009), canola (Thiessen et al., 2004; Sha-faeipour et al., 2008), lupin (Glencross et al., 2005, 2006) and soybean products (Cheng et al., 2003; Chou et al., 2004), are under study. Factors such as level of inclusion (Kaushik et al., 1995; Gomes et al., 1995; Carter and Hauler, 2000), ingredient digestibility (Aksnes et al., 2006; Romarheim et al., 2008), effects of the presence of different anti-nutritional factors (Halver and Hardy, 2002;

Gatlin et al., 2007; Iwashita et al., 2008), nutrient supplementation (Sugiura et al., 2001; Cheng et al., 2003; Hernandez et al., 2005), intestinal inflammatory responses (Heikkinen et al., 2006; Iwashita et al., 2008; Yamamoto et al., 2008) and ingredient processing (Vielma et al., 2000; Barrows et al., 2007; 2008) have been investigated by researchers around the world to evaluate performance of carnivorous fish when fed diets with various plant meal products. All these research have advanced our understanding about how plant meal can be used for FM replacement, but this task has proven to be more complex than expected.

A main scientific research area in fish feed production is to replace FM with plant protein sources. However, FM cannot be totally replaced by plant protein sources without significant reduction in fish performance or feed utilization for most species investigated. Even when experimental diets meet the known requirements of nutrients for fish, they still generally perform poorer than high FM diets partly (Gaylord et al., 2006; Lim and Lee, 2009). The reduced growth performance by fish fed plant protein sources is partially explained by the presence of anti-nutritional factors (Olli et al., 1994; Francis et al., 2001). In addition, other differences between protein sources of plant and marine origin could also be of importance. For instance, taurine has been shown to improve growth in oilve flounder (Park et al., 2001; Kim et al., 2005), redsea bream (Matsunari et al., 2008), cobia (Lunger et al., 2007), yellowtail (Matsunari et al., 2005), common dentex (Chatzifotis et al., 2008) and rainbow trout (Gaylord et al., 2006). Taurine is present in marine and animal feed stuffs, but mainly absent in plant resources (Aksnes, 2006).

1.3. Nutritional limitations of plant products

1.3.1. Amino acid limitations

Soybean protein is well known to be limiting in total sulphur amino acids (TSAA; methionine plus cysteine) when utilized in animal feeds. Soybean meal and soy protein concentrate each contain TSAA at approximately 2.95% of protein. Supplemental methionine can overcome part of the growth reduction observed when a large percentage of the total dietary protein is from soy origin (Cai and Burtle, 1996; Keembiyehetty and Gatlin, 1997; Yamamoto et al., 2002). Additional supplementation with lysine also improved growth indicating that it may be another limiting one as well (Floreto et al., 2000; Furuya et al., 2004). Other sulphur-containing compounds that are metabolic derivatives of methionine and cysteine also may be limiting in aquafeeds when plant proteins are the primary protein source. Most of plant products do not contain taurine, which has been demonstrated to be conditionally indispensable for some fish species (Takeuchi, 2001). Current research also has indicated that taurine may be limiting in all-plant protein diets, even for rainbow trout, which have some capacity to synthesize taurine from cysteine.

1.3.2. Phytic acid

Approximately two-thirds of the total phosphorus in oilseed meals or grains and their by-product meals is present as phytic acid (phytate), the main storage form of phosphorus in seeds. It cannot be digested and absorbed by monogastric animals including fish (Barual et al., 2004). Furthermore, phytic acid lowers the availability of certain divalent cations, notably zinc, to carnivorous species of fish and to omnivorous species, and also has been reported in some studies to reduce the apparent digestibility of protein. Heat treatment associated with extrusion pelleting does not improve the availability of phytate phosphorus in oilseed meals or grains or reduce antagonistic interactions with other essential nutrients. The only effective strategies to reduce the effects of phytic acid are to remove phytic acid by processing, hydrolyse it using the

enzyme phytase or to utilize single-gene mutant varieties of grains and oilseeds in which a smaller percentage of total phosphorus in the seed is stored as phytate phosphorus.

1.3.3. Protease inhibitors

Legume seeds, as many other plant seeds, contain one or more protease inhibitors that will inhibit proteolytic enzymes in the gastrointestinal tract of pests that commonly attack the seeds in fields (Liener, 2000). As digestive proteases have been highly conserved through evolution, the plant protease inhibitors such as trypsin inhibitors also inhibit proteases in the gastrointestinal tract of monogastric animals. Enzymes of fish seem to be particularly sensitive to the protease inhibitors (Krogdahl and Holm, 1983). Heat treatment will inactivate protease inhibitors to a major extent. However, the heat needed for total inactivation of the inhibitors may reduce protein quality through oxidation and fusion with other components of the plant proteins. A compromise must therefore be made between inactivation and deterioration of protein quality, which may leave some inhibitors active.

1.3.4. Lectins

One of several antinutritional factors found in plant feedstuffs are the bioactive group of glycoproteins known as lectins (NRC, 1993; Francis et al., 2001). Owing to their ability to agglutinate red blood cells they are also referred to as agglutinins. Lectins possess the ability to bind reversibly and specifically to carbohydrates and glycoconjugates, which is responsible for their numerous physiological effects. Lectins easily avoid digestion and then pass into the intestine where they may bind with the epithelium. Some lectins may cause disruption of membrane integrity and the initiation of a cascade of immune and autoimmune events that ultimately lead to cell death. Lectins may also increase permeability of membranes to other

proteins increasing incidence of allergic reactions. In addition to the disruption of intestinal membrane integrity, lectins such as SBA (Soybean agglutinins) have been observed in some cases to cause hypertrophy, hyperplasia, decreased nutrient uptake, decreased intestinal enzyme activity, increase gut length and mass as well as increase pancreatic secretions. Lectins may have an effect on systemic insulin levels and may mimic its effects. Lectins are resistant to proteolytic enzymes, but are either inactivated or destroyed by heat treatment. They are not affected by heating to 60℃ but are destroyed when heated to 100℃ for 5min.

1.3.5. Saponins

In general, saponins have high toxicity to fish when applied externally (Roy et al., 1990) and preparations of saponin extracts from tea seed cake have been used to eradicate predacious fish in ponds (Chen and Chen, 1997). The majority of work on saponins has concentrated on *Quillaja* saponin (derived from the *Quillaja saponaria* Molina tree) because of its use in vaccine adjuvants (Oda et al., 2003). *Quillaja* saponins were recognized for their toxicity; however, supplementation of 0.6% cholesterol into a diet for chicks with 0.6% *Quillaja* saponins restored feed intake and growth of animals. Makkar and Becker (2005) examined the role of dietary *Quillaja* saponin addition on performance, growth and fecundity of tilapia and common carp. It was apparent that control fish showing depressed growth was directly associated with frequent mouth-brooding episodes during experiment, whereas in other treatments with less females, fish did not spawn any eggs. Therefore it can be concluded that growth data were not representative for group-feeding tilapia and confounded by an uneven sex ratio in treatments and reproduction of females. Therefore, at this time it can be concluded that there is no substantial, scientifically sound evidence to suggest any positive role of *Quillaja* saponins in fish nutrition.

1.3.6. Carbohydrate fractions

Plant proteins are characterized by a high content of NSPs (Non-starch polysaccharides) and negligible starch. The high NSPs content represents a major challenge for the use of plant protein products in fish diets. This fraction provides marginal energy for the fish due to limited microbial fermentation, and may negatively affect nutrient utilization and thereby reduce feed efficiency. The antinutritional actions of soluble NSPs are still not fully understood.

1.3.7. Gossypol

Gossypol ($C_{30}H_{30}O_8$) is a toxic polyphenolic pigment present in cotton plant (*Gossipium* spp.) and the main limiting factor in cottonseed meal containing diets for most monogastric animals including fish. Gossypol toxicity depends on several factors including the form of gossypol ((+)- or (-)-enantimer), the consumed amounts or varieties of the cottonseed species. Furthermore, toxic effects of the gossypol are reported to be much greater in non-ruminants including fish than ruminants (Willard et al., 1995). To remove gossypol from cottonseed, a number of methods have been attempted including solvent extraction (Canella and Sodini, 1977; Cherry and Gray, 1981), chemical treatment with ferrous sulfate (Barraza et al., 1991; Lim and Lee, 2008) or calcium hydroxide (Nagalakshmi et al., 2002, 2003) and microbial fermentation (Zhang et al., 2006, 2007).

1.4. Taurine

Taurine (2-aminoethanesulfonic acid) is a beta-amino sulfur amino acid, but it is neither an

essential amino acid in most animals nor a building block of proteins. It is the major free intracellular amino acid that produces oxidants and toxic substances in many tissues, including the brain, retina, myocardium, skeletal muscle, liver, platelets and leukocytes (Chesney, 1985, Wright et al., 1986). The main source of taurine for most mammals is the diet, although some species are capable of surviving without much harm. In mammals, various physiological functions have been attributed to taurine. However, in fish, much has to be understood about the role that taurine plays in the maintenance of life and normal function.

1.5. Taurine biosynthesis

Taurine is the most abundant amino acid in most of animal tissues and can be obtained or synthesized from cysteine in the body. In mammals, taurine can be synthesized by 2 pathways (1): the conversion of cysteine to cysteinesulfinate (CSA) by cysteine dioxygenase (CDO), followed by its decarboxylation to hypotaurine by cysteinesulfinic acid decarboxylase (CSD) and the oxidation of hypotaurine to taurine (Fig. 1-1) and (2) the incorporation of cysteine into CoA, followed by the release of cysteamine during CoA turnover, the oxidation of cysteamine to hypotaurine by cysteamine (2-aminoethanethiol) dioxygenase (ADO) and the further oxidation of hypotaurine to taurine.

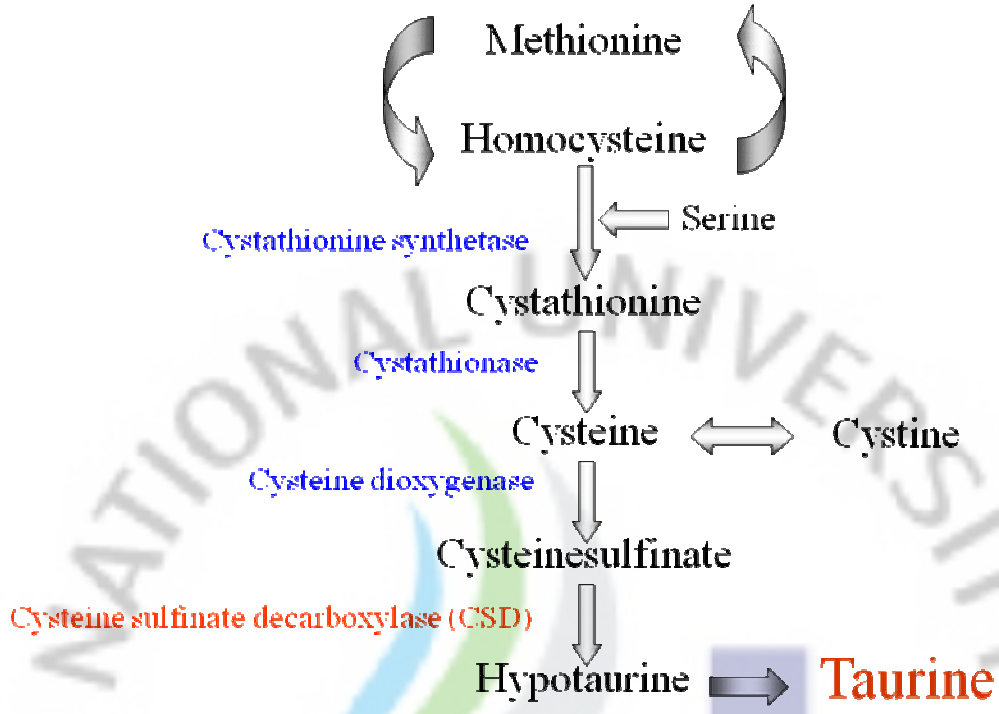

Fig. 1-1. Metabolic pathway of sulfer amino acid

(Source: Park et al., 2002 in Aquaculture)

1.6. Hepatic CSD activity

The potential pathways of taurine synthesis are reviewed elsewhere (Oja and Kontro, 1983). The predominant route of synthesis varies among species and depends on types of tissue. However, the enzyme CSD appears to be the rate-limiting step in taurine biosynthesis in many mammalian species (Jacobsen and Smith, 1968). Cats have low activity of CSD, the rate-limiting enzyme for taurine biosynthesis, and are dependent on a dietary source of taurine (Knopf et al., 1978; Sturman et al., 1986). Human infants, children, and adults who were fed totally with parenteral nutrition (via intravenous administered fluids) had reduced concentrations of taurine in their plasma, since such solutions did not contain taurine (Geggel et al., 1985; Vinton et al., 1987; Zelikovic et al., 1990). In fish, activity of this enzyme varies depending upon species and size. For example, in the yellowtail as well as in bluefin and skipjack tunas, CSD activity is not present, whereas in olive flounder it expresses only low activity (Yokoyama et al., 2001; Fig. 1-2).

1.7. Biological function of taurine

In mammals, taurine is known to serve many important biological functions including cell membrane stabilization (Pasantes-Morales et al., 1985), antioxidation (Nakamura et al., 1933), detoxification (Huxtable, 1992), osmoregulation (Thurston et al., 1980), neuromodulation (Bernardi, 1985) and brain and retinal development (Sturman, 1986). Recent work has indicated that taurine is conditionally indispensable for some carnivorous species (Takeuchi, 2001) and will support increased growth when supplemented to plant protein based diets (Gaylord et al.,

2006). Taurine also has been determined to be conditionally indispensable for olive flounder (Takeuchi, 2001) and a taurine deficiency can cause green liver syndrome in red sea bream (Goto et al., 2001). In addition, taurine supplementation to the soy protein concentrate based diet dramatically improved the physiological state of yellowtail. In teleost, taurine is the sole amino acid that conjugates with cholic acid to produce bile salts (Kim et al., 2008). Although taurine is a well known constituent in fish, there is little information available on its applications to aquaculture.

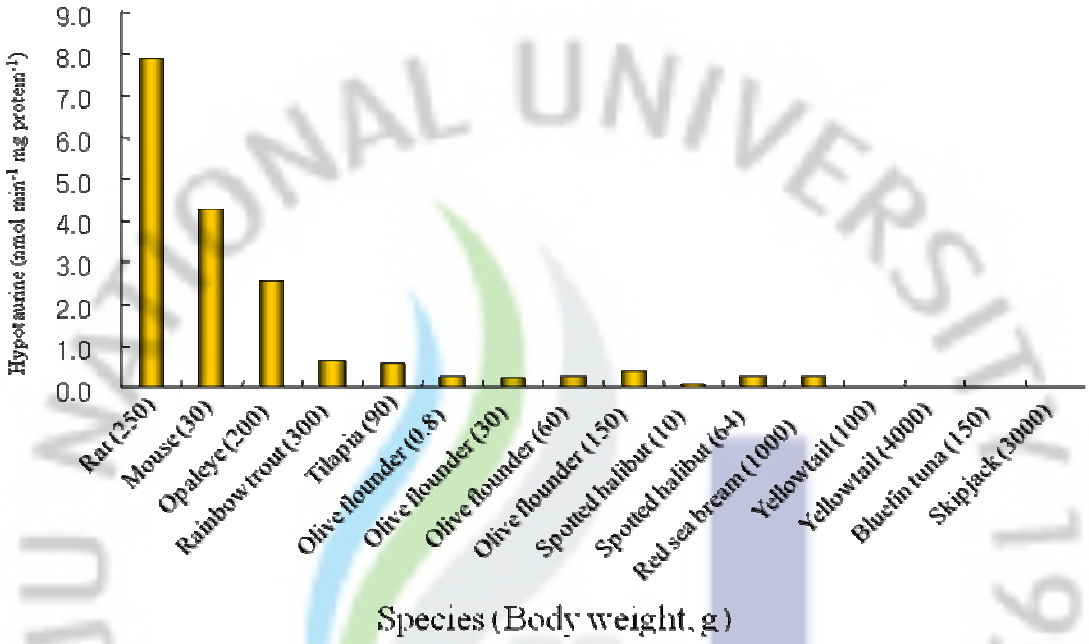

Fig. 1-2. Hapatic CSD activity

(Source: Yokoyama et al., 2001 in Aquaculture Research)

1.8. Effect of dietary taurine in fat digestion

In lipid metabolism, the function of taurine is considered because of its conjugation with bile acids in the liver (Danielsson, 1963), which increase the use of bile acids, the degrading metabolites of cholesterol and that participate in the formation of micelles that are used for fat absorption in the small intestine (Yamanaka et al., 1986). Conjugated bile acid is stored in the gall bladder, and finally is released into the intestine. Bile acids have a surfactant function in emulsifying fats to make them more accessible for absorption. Bile insufficiency results in undigested fats being passed in the feces, a condition known as steatorrhea in humans (Heaton, 1972). In mammals, the most common bile acids are either taurocholic acid or glycocholic acid, with the latter being found only in placental mammals. Taurocholic acid is a more efficient bile acid in fat absorption, because taurine-conjugated bile acids are better fat emulsifiers than glycine-conjugated bile acids (Chesney et al., 1998). In all vertebrates except for mammals, taurine is the sole amino acid conjugated with cholesterol derivatives to form bile salts. In fish, bile acid is conjugated not with glycine but with taurine by the bile acid coenzyme A (CoA)–amino acid N-acyltransferase in the liver of fish (Vessey et al., 1990). This implies that taurine can be the sole amino acid for conjugated bile acid that is involved with lipid digestion and absorption in fish (Fig. 1-3).

1.9. Estimated dietary taurine requirement

Recent investigations have indicated that taurine is an essential element for larval and juvenile marine finfish (Takeuchi et al., 2001; Yokoyama et al., 2001). For example,

supplementation of taurine in the diet improves the growth of the juvenile olive flounder (Park et al., 2002; Kim et al., 2003) and taurine enrichment of rotifers is effective to improve the growth and survival ability in red sea bream larvae (Chen et al., 2004). Dietary taurine requirements in juvenile stages of marine fish have been reported in olive flounder (1.5–2.0%), yellowtail (>1.0%) and European sea bass (0.2%). In addition, Takagi et al. (2006) reported that the occurrence of green liver induced by highly inclusion of soybean meal in yearling red sea bream was prevented by the supplementation of taurine. However, dietary taurine requirement of parrot fish has been determined neither in juvenile stage nor young or adult stages.

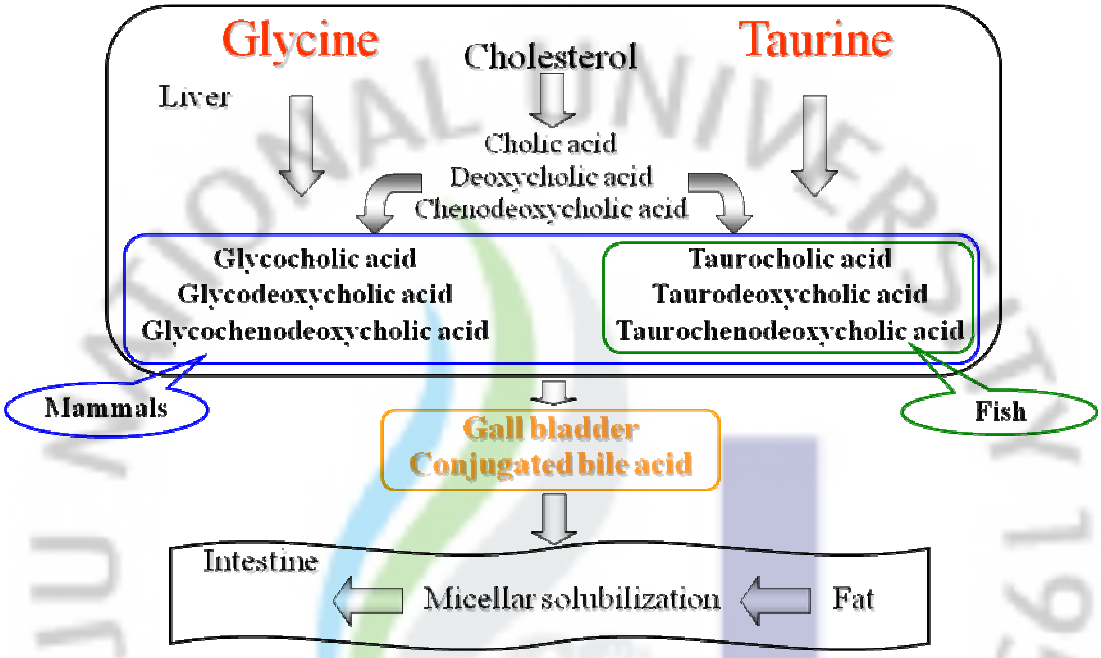

Fig. 1-3. Metabolic pathway of conjugated bile acids

(Source: Kim et al., 2008 in Fisheries Science)

1.10. Chapter Justification

The studies presented in this dissertation are aimed at analyzing the effects of dietary taurine supplementation on nutritional and physiological performance of parrot fish, and to determine whether taurine supplementation to plant protein based diet would improve growth performance of juvenile or ongrowing parrot fish. All chapters are focused on the effects of dietary taurine supplementation on feed utilization and growth performance. The justification of each chapter is as follows;

Chapter 2 is about the utilization of plant protein sources for FM replacement in diets of juvenile and ongrowing parrot fish. In this study, two consecutive feeding trials were conducted to determine the optimum dietary level of cottonseed and soybean meal (CS) for replacement of FM in diets for juvenile and ongrowing parrot fish with or without iron and phytase.

Chapter 3 was conducted to determine whether the taurine supplementation to soybean meal based diet would improve growth performance of juvenile parrot fish. In this study, experimental diets were supplemented with taurine at 0 and 1.0% to a fish meal based diet (designated as FM or FM+T, respectively), and with taurine at 0, 0.05, 1.0 and 2.0% to a soybean meal based diet (replacement of 30% fish meal protein), respectively (designated as SM, SM+TL, SM+TM or SM+TH, respectively).

Chapter 4 focuses on the supplemental effects of taurine in ongrowing parrot fish fed diets containing high levels of soybean meal protein. .

Chapter 5, finally, focuses on the taurine essentiality or requirement on dietary taurine

concentration, feed utilization and growth performance for juvenile parrot fish.

CHAPTER TWO

Use of cottonseed and soybean meal in diet for parrot fish (*Oplegnathus fasciatus)*

2.1. Introduction

Fish meal (FM) is a major protein source in aquafeeds especially for carnivorous fish species because it is an excellent source of essential nutrients such as indispensable amino acids, essential fatty acids, vitamins, minerals, attractants and unknown growth factors (Zhou et al., 2004). However, increasing demand, unstable supply and high price of the FM with the expansion of aquaculture made it necessary to search for alternative protein sources (FAO, 2004; Lunger et al., 2007).

Defatted soybean meal (SM) has been the most frequently studied ingredient as a FM replacer in diets for many fish species, due to its high protein content, relatively well-balanced amino acid profile, reasonable price and steady supply (Storebakken et al., 2000). Several studies have shown promising SM results in aquafeed formulation for carnivorous and herbivorous fish species. Data have shown that approximately 20 to 40% FM protein can be replaced in diets for carnivorous fish species (Chou et al., 2004; Lim et al., 2004; Hernandez et al., 2007; Pham et al., 2007).

Cottonseed meal (CM) has long been used in diets for both terrestrial animals (Colin-Negrete et al., 1996) and fish (Hendricks et al., 1980) because of its high protein content, availability and low cost. CM has been tested in numerous fish species such as rainbow trout

(Cheng and Hardy, 2002), channel catfish (Robinson and Li, 1994), tilapia (El-Sayed, 1990; Mbahinzireki et al., 2001), largemouth bass (Kurten et al., 1999) and sunshine bass (Rawles and Gatlin, 2000). However, when compared to FM, a mixture of CM and SM (CS) may result in several problems including lower crude protein level, suboptimal levels of certain essential amino acids, palatability issues and the presence of antinutritional factors (NRC, 1993; Storebakken et al., 2000; Imorou et al., 2007). Phytic acid content is the main limiting factor in using plant protein sources in diets for most monogastric animals including fish. Most plant protein sources used in fish diets contain phytic acid in the range of 5 to 30 g kg^{-1} (Reddy, 2002) and approximately 70% of their total phosphorus content is bound as phytate (Lall, 1991) which is not available for fish. Supplemental phytase (Biswas et al., 2007; Pham et al., 2008) has been used to liberate free phosphorus from phytate (Albrektsen et al., 2006; Lim and Lee, 2008) in diets containing plant protein sources. Additionally, CM contains gossypol which is toxic to fish (Herman, 1970) leading to a restriction of its use as a fish feed ingredient.

Parrot fish, a subtropical marine fish, is carnivorous species and has been regarded as an emerging aquaculture species because of its high economic value, excellent meat quality and strong resistance to diseases. However, nutritional information on this species is limited (Kang et al., 1998; Wang et al., 2003) and no data are available on the dietary utilization of the CS. We have recently reported that CS with iron and phosphorus supplementation could replace up to 40% FM protein in diets for juvenile olive flounder in long-term feeding trial of over 6 months (Lim and Lee, 2008). That study indicated a potentiality of CS for FM replacement in diets for carnivorous marine fish species. Therefore, two consecutive feeding trials were conducted to determine the optimum dietary level of the CS with or without iron and phytase in the presence of supplemental lysine and methionine for the FM replacement in two different growth phases of parrot fish.

2.2. Materials and methods

Two consecutive feeding trials were conducted to determine the optimum dietary inclusion level of an equal mixture of cottonseed and soybean meal (CS) for replacement of FM in diets for juvenile (experiment I) and ongrowing (experiment II) parrot fish with or without iron and phytase.

2.2.1. Experimental diets

In experiment I, six experimental diets were formulated to replace FM protein by equal proportion (1:1, w:w) of cottonseed and soybean meal (CS) at 0, 10, 20, 30, 40, or 50% (designated as CS0, CS10, CS20, CS30, CS40, or CS50, respectively). The CS containing diets were supplemented with L-methionine and L-lysine to meet their estimated dietary requirements (NRC, 1993). The dietary formulation, proximate composition and gossypol content are presented in Table 2-1. All diets were formulated to be isonitrogenous (46% crude protein) and isocaloric (22 MJ/kg diet). Solvent extracted cottonseed meal was provided by Southern Cotton Oil Co., Memphis, TN, USA. Its protein and fiber content were 43.5% and <12% on a dry matter basis, respectively. All dry materials were thoroughly mixed with 30% double distilled water, extruded through a meat chopper machine (SMC-12, Kuposlice, Busan, Korea) at 5 mm in diameter, freeze-dried at -40 ^{0}C for 24 h and stored at -20 ^{0}C until use.

In experimental II, diets were formulated to replace FM protein by equal proportion (1:1, w:w) of CS at 0, 20, or 30% (designated as CS0, CS20, or CS 30, respectively) and formulated with supplementation of ferrous sulfate (0.1 and 0.2%) and phytase in the CS20 and CS30 diets (designated as CS20+Fe&P and CS30+Fe&P). Microbial phytase at 1000 FTU kg diet^{-1} was

used in the diets as described by Cheng and Hardy (2003) and Yoo et al. (2005). The CS containing diets were also supplemented by L-methionine and L-lysine, the same levels as in the experiment I (Table 2-2). The diets were formulated to be isonitrogenous (46% crude protein) and isocaloric (22 MJ kg diet^{-1}).

Table 2-1. Formulation and proximate composition of diets used in experiment I (% dry matter)

Ingredients	Diets					
	CS0	CS10	CS20	CS30	CS40	CS50
White fish meal	52.0	46.8	41.6	36.4	31.2	26.0
Soybean meal	0.0	3.8	7.6	11.5	15.3	19.1
Cottonseed meal[a]	0.0	4.0	8.1	12.1	16.1	20.1
Corn gluten meal	8.0	7.7	7.4	7.1	6.8	6.5
Wheat flour	6.5	6.5	6.5	6.5	6.5	6.5
Starch	16.0	13.7	11.4	9.1	6.8	4.5
Yeast	2.0	2.0	2.0	2.0	2.0	2.0
Mineral mix[b]	1.0	1.0	1.0	1.0	1.0	1.0
Vitamin mix[c]	1.0	1.0	1.0	1.0	1.0	1.0
Squid liver oil	11.0	11.3	11.5	11.7	12.0	12.3
Lysine[d]	0.0	0.1	0.2	0.3	0.4	0.5
Methionine[e]	0.0	0.1	0.2	0.3	0.4	0.5
Cellulose	2.5	2.0	1.5	1.0	0.5	0.0
Chemical analyses						
Crude protein, % DM	46.3	46.1	46.3	46.4	46.5	46.8
Crude fat, % DM	16.1	15.4	15.6	16.0	16.2	16.9
Ash, % DM	7.9	7.8	7.5	7.3	7.2	7.2
Fiber, % DM[f]	1.1	1.8	2.5	3.2	3.9	4.6
NFE, % DM[g]	28.6	28.9	28.1	27.1	26.2	24.5
Gross energy, MJ/kg DM[h]	22.2	21.9	21.9	21.9	21.9	21.9
Total gossypol (ug g^{-1})[i]	nd[j]	316	507	858	1016	1274
(+)-Enantiomer	nd	192	317	528	623	783
(-)-Enantiomer	nd	124	190	330	393	491

[a] Cottonseed meal was purchased from Southern Cotton Oil Co., Memphis, TN, USA

[b] Mineral premix (g kg^{-1} mixture) $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; CuCl, 0.2; $AlCl_3$. $6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[c] Vintamin premix (g kg^{-1} mixture) L-ascorbic acid, 121.2; DL-α tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-$_D$-pantothenate, 12.7; myo-inositol, 181.8; $_D$-biotin, 0.27; folic acid, 0.68; p-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003.

[d] L-lysine mono-hydrochloride, Sigma, USA

[e] L- methionine, Sigma, USA

[f] Fiber content was calculated based on fiber contents of white fish meal, soybean meal, cottonseed meal, corn gluten meal, wheat flour and yeast.

[g] Nitrogen-free extract (NFE) = 100-(% protein + % lipid + % ash + % fiber)

[h] Gross energy of experimental diets was calculated according to gross energy values 5.64 kcal/g crude protein, 4.11 kcal/g carbohydrate, and 9.44 kcal/g crude fat, respectively (NRC, 1993).

[i] Total gossypol includes free and bound gossypol

[j] nd: not detected

Table 2-2. Formulation and proximate composition of diets used in experiment II (% dry matter)

Ingredients	Diets				
	CS0	CS20	CS30	CS20 +Fe&P	CS30 +Fe&P
White fish meal	52.0	41.6	36.4	41.6	36.4
Soybean meal	0.0	7.7	11.5	7.7	11.5
Cottonseed meal[a]	0.0	8.1	12.1	8.1	12.1
Corn gluten meal	8.5	7.9	7.6	7.9	7.6
Wheat flour	6.5	6.5	6.5	6.5	6.5
Starch	18.3	13.6	11.3	13.6	11.3
Mineral mix[b]	1.0	1.0	1.0	1.0	1.0
Vitamin mix[c]	1.0	1.0	1.0	1.0	1.0
Squid liver oil	11.0	11.5	11.8	11.5	11.8
Lysine[d]	0.0	0.2	0.3	0.2	0.3
Methionine[e]	0.0	0.2	0.3	0.2	0.3
Ferrous sulfate $7H_2O$	0.0	0.0	0.0	0.1	0.2
Phytase[f]	0.0	0.0	0.0	0.01	0.01
Cellulose	1.7	0.7	0.2	0.6	0.0
Chemical analyses					
Crude protein, % DM	46.0	45.7	45.8	45.5	46.1
Crude fat, % DM	15.8	16.0	16.1	15.9	15.9
Ash, % DM	8.0	7.5	7.3	7.6	7.8
Fiber, % DM[g]	1.1	2.4	3.1	2.4	3.1
NFE, % DM [h]	29.1	28.4	27.7	28.6	27.1
Gross energy, MJ kg^{-1} DM[i]	22.1	22.0	21.9	21.9	21.8
Total gossypol (mg/kg)[j]	nd[k]	461	661	439	695
(+)-Enantiomer	nd	319	461	303	477
(-)-Enantiomer	nd	142	200	136	218

[a] Cottonseed meal was purchased from Southern Cotton Oil Co., Memphis, TN, USA

[b] See the footnote in table 2-1

[c] See the footnote in table 2-1

[d] L-lysine mono-hydrochloride, Sigma, USA

[e] L- methionine, Sigma, USA

[f] Phytase (10,000 FTU/g) was purchased from Easy Bio System, Inc., Seoul, Korea.

[g] Fiber content was calculated based on fiber contents of white fish meal, soybean meal, cottonseed meal, corn gluten meal and wheat flour.

[h] Nitrogen-free extract (NFE)= 100-(% protein + % lipid + % ash + % fiber)

[i] Gross energy of experimental diets was calculated according to gross energy values 5.64 kcal/g crude protein, 4.11 kcal/g carbohydrate, and 9.44 kcal/g crude fat, respectively (NRC, 1993).

[j] Total gossypol includes free and bound gossypol

[k] nd: not detected

2.2.2. Feeding trials and sample collection

Juvenile parrot fish (*Olegnatlus fasciatus*) were transported from a private hatchery (Jeju Island, Korea) to our laboratory. The fish were fed a commercial diet (Suhyupfeed Co., Ltd, GyeongNam, Korea) for 2 weeks to acclimate to the experimental conditions. In experiment I, 20 fish (IBW, 3.17±0.01 g/fish) were randomly distributed into 18-60 L polyvinyl circular tanks. The tanks were supplied with filtered seawater at a flow-rate of 2.5 L/min and aeration to maintain a proper level of dissolved oxygen. Water temperature ranged from 17 to 21℃ according to seasonal change. Triplicate groups of fish were fed the experimental diets to apparent satiation (twice a day, 09:00 and 17:00 h) for 12 weeks. Uneaten feed was collected 30 min after feeding and reweighed to determine feed intake. Growth of fish was measured every 3 weeks. Feeding was stopped 24 h prior to weighing to minimize stress of the fish.

At the end of feeding trial, three fish per tank (9 fish per dietary treatment) were randomly sampled and anaesthetized with MS-222 solution (200 mg L^{-1}) for blood analyses. Blood samples were taken from the caudal vein with heparinized syringes. Livers from three fish per tank were removed and stored at -80 ^{0}C for analysis of gossypol.

In experiment II, after a 2 week conditioning period, parrot fish with an initial body weight of 55±0.5 g (mean ± S.D.) were distributed to each tank as groups of 20 fish per tank and fed one of the five experimental diets to apparent satiation (twice a day, 09:00 and 17:00 h) for 9 weeks. The tanks were supplied with filtered seawater at a flow-rate of 3-4 L/min and aeration was used to maintain optimum dissolved oxygen level. Water temperature ranged from 22 to 27℃ according to the seasonal change. Weight of the fish was measured every 3 week. Feeding was stopped 24 h prior to weighing. Sample collections for analysis of blood parameters and

liver gossypol were as described for experiment I.

2.2.3. Analysis

At the end of the feeding trials, all fish in each tank were weighed and counted to compute the weight gain, feed conversion ratio, specific growth rate, protein efficiency ratio and survival. Hematocrit was determined for three individual fish per tank by a microhematocrit technique (Brown, 1980). Hemoglobin, plasma tryacyglyceroles and total cholesterol were determined in the same three fish by using the automated blood analyzer (SLIM, SEAC Inc, Florence, Italy). Analyses of crude protein, moisture and ash in the diets were performed by standard methords (AOAC, 1995). Dietary lipid was determined by the method of Folch et al. (1957).

Total gossypol in diets and liver (9 fish per treatment) was determined by High Performance Liquid Chromatography (HPLC) by the method described by Lee and Dabrowski (2002). The liver and dry diets were weighed and 3 - 10 volumes of reagent mixture were added to obtain the 2-amino-1-propanol derivatives of gossypol. The reagent mixture was composed of 2 mL 2-amino-1-propanol (Sigma Chemical, St. Louis, MO), 10 mL glacial acetic acid (Sigma Chemical) and 88 mL N, N-dimethylformamide (Sigma Chemical). The samples were homogenized in the reagent mixture for 30 sec, heated at 95 ^{o}C for 30 min, cooled on ice and then centrifuged at 1500 x g for 5 min. After centrifugation, an aliquot of the supernatant was diluted with the mobile phase [800 mL acetonitrile add 10 mM KH_2PO_4 dissolved in 200 mL water (HPLC grade) adjusted to pH 3.0 with H_3PO_4] to obtain a desirable concentration, centrifuged again at 1500 x g for 5 min and filtered through a syringe filter (0.45 μm; Whatman Inc., Clifton, NJ, USA) before injection to HPLC. The mobile phase was delivered at a flow-rate

of 1.0 mL/min. The HPLC injection volume was 20 μL. The retention time for the (+)- and (-)-gossypol were 3.5 and 5.6 min, respectively.

Total polyphenolic compounds in the experimental diets were measured by a colorimetric method as described by Skerget et al. (2005).

2.2.4. Statistical analysis

All diets were assigned by a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the difference in means were made with Duncan's multiple range test. Statistical significance was determined by setting the aggregate type I error at 5% ($P<0.05$) for each set of comparisons. Data are presented as means±SD. Percentage data were arcsine transformed before statistical analysis.

2.3. Results

2.3.1 Experiment I

The fish readily accepted all diets and no fish died during the feeding trial. At the end of 12 weeks of the feeding trial, negative effects on growth performance were observed when 30% of FM protein was replaced by CS protein. Significant differences were found in weight gain, feed intake, feed conversion ratio, specific growth rate and protein efficiency ratio when the replacement level for FM protein was increased from 20 to 30% (Table 2-3).

Groups of fish fed CS50 diet had significantly lower hematocrit than fish fed the control CS0 diet (Table 2-4). However, hemoglobin values were not different among the fish groups. The dietary supplementation of CS significantly reduced the level of plasma triacyglycerols and total cholesterol (Table 2-4).

Dietary gossypol content was increased with each increment of CM incorporation in the diets. At the end of the feeding trial, total and each (+) and (-) gossypol enantiomer concentration in the livers were slightly increased as the CM inclusion increased in the diets (Table 2-5). However, liver gossypol (both total or each enantiomer) was not detected in fish fed CS10 diet.

2.3.2. Experiment II

The second experiment resulted in no differences in growth performances or utilization of the experimental diets and no fish died during the feeding trial (Table 2-3).

Hematocrit values were significantly lower in fish fed the CS containing diets without iron and phytase supplementation than that of fish fed the FM based control diet. However, the values in fish fed the CS containing diets with iron and phytase supplementation were not different compared to that of fish fed the control diet (Table 2-4). Hemoglobin values did not differ among the fish groups. The results of plasma triacyglycerol and cholesterol levels followed a similar trend as the results of experiment I (Table 2-4).

Dietary and liver gossypol concentrations were also similar to those observed in experiment I (Table 2-5). Total and each (+) and (-) gossypol concentrations in the liver were slightly increased as the CM inclusion increased in the diets. Interestingly, however, liver gossypol of fish fed the CS containing diets (CS20+ Fe&P, CS30+Fe&P) supplemented with iron (as ferrous sulfate at 0.1 and 0.2%, respectively) was not detected. This result indicates that the addition of iron in diets containing cottonseed meal prevents the absorption of free gossypol.

Table 2-3. Growth performance of juvenile (IBW, 3.2±0.01g) and ongrowing (IBW, 55±0.5g) parrot fish in the two feeding trials fed different experimental diets for 12 and 9 weeks, respectively

Experiment I (juvenile parrot fish)						
Diets	CS0	CS10	CS20	CS30	CS40	CS50
WG (%)[a]	617±32.7[a]	616±26.9[a]	603±21.9[a]	529±32.6[b]	520±26.7[b]	465±63.5[c]
FCR[b]	1.11±0.06[a]	1.15±0.04[a]	1.15±0.02[ab]	1.25±0.04[bc]	1.28±0.09[cd]	1.44±0.12[d]
SGR (%)[c]	1.96±0.11[a]	1.90±0.07[ab]	1.89±0.03[ab]	1.74±0.06[bc]	1.70±0.11[c]	1.51±0.12[d]
PER[d]	1.02±0.02[a]	1.02±0.02[a]	1.01±0.02[a]	0.95±0.03[b]	0.94±0.02[b]	0.89±0.03[c]

Experiment II (ongrowing parrot fish)					
Diets	CS0	CS20	CS30	CS20+Fe&P	CS30+ Fe&P
WG (%)[a]	111±10.8	104±10.1	111±7.2	121±0.7	115±9.2
FCR[b]	1.72±0.15	1.81±0.23	1.72±0.09	1.61±0.03	1.69±0.11
SGR (%)[c]	1.28±0.09	1.22±0.11	1.28±0.07	1.33±0.05	1.27±0.09
PER[d]	1.30±0.11	1.24±0.15	1.29±0.07	1.38±0.03	1.32±0.09

Means of triplicate groups, values are presented as mean ±SD. Values in the same row having different superscript letters are significantly different ($P < 0.05$).

[a] Weight gain (%) = 100 x (final mean body weight - initial mean body weight) x initial mean body weight^{-1}

[b] Feed conversion ratio = dry feed fed x wet weight gain^{-1}

[c] Specific growth rate (%) = [(loge final body weight - loge initial body weight) x days^{-1}] x 100

[d] Protein efficiency ratio = wet weight gain x total protein given^{-1}

Table 2-4. Blood parameters of juvenile and ongrowing parrot fish in the two feeding trials fed different experimental diets for 12 and 9 weeks, respectively

Experiment I (juvenile parrot fish)						
Diets	CS0	CS10	CS20	CS30	CS40	CS50
Hematocrit (%)	45.6±1.0^{a}	42.7±3.5^{ab}	43.3±3.0^{ab}	41.8± 0.2^{ab}	41.8±2.8^{ab}	39.8±1.6^{b}
Hemoglobin (g/dL)	10.0±0.5	9.9±0.5	9.8±0.9	9.6±0.7	9.5±1.4	9.3±1.7
Triacyglycerol (mg/dL)	108±10.6^{a}	68±14.6^{b}	75±13.3^{b}	70±21.7^{b}	68±3.4^{b}	63±5.3^{b}
Cholesterol (mg/dL)	274±17.9^{a}	234±0.3^{b}	199±10.1^{b}	191±15.0^{b}	202±17.2^{b}	198±6.0^{b}

Experiment II (ongrowing parrot fish)					
Diets	CS0	CS20	CS30	CS20+Fe&P	CS30& Fe&P
Hematocrit (%)	45.6±0.8^{a}	40.4±1.7^{b}	40.1±2.3^{b}	44.0±1.9^{a}	43.8±3.1^{a}
Hemoglobin (g/dL)	8.7±0.8	7.5±0.6	7.6±0.5	7.9±0.7	8.1±1.6
Triacyglycerol (mg/dL)	51.9±16.7^{a}	25.7±8.6^{b}	27.6±9.7^{b}	35.5±5.4^{ab}	32.4±1.6^{b}
Cholesterol (mg/dL)	140±27.1^{a}	88±31.8^{b}	78±10.1^{b}	65±10.5^{b}	89±27.6^{b}

Mean values of triplicate groups, values are presented as mean ± SD. Values in the same row having different superscript letters are significantly different ($P < 0.05$).

Table 2-5. Total (+ and -) gossypol enantiomer accumulation in liver of juvenile and ongrowing parrot fish in the two feeding trials fed different experimental diets for 12 and 9 weeks, respectively (ug g wet weight^{-1})

	(+)-enantiomer	(-)-enantiomer	Total
Experiment I (juvenile parrot fish)			
CS0	nd	nd	nd
CS10	nd	nd	nd
CS20	2.1±0.57^{a}	0.8±0.15^{a}	2.9±0.72^{a}
CS30	3.2±0.12^{b}	1.2±0.04^{b}	4.4±0.16^{b}
CS40	3.7±0.77^{b}	1.5±0.11^{c}	5.2±0.88^{b}
CS50	5.0±0.55^{c}	2.3±0.13^{d}	7.3±0.68^{c}
Experiment II (ongrowing parrot fish)			
CS0	nd	nd	nd
CS20	2.2±0.44^{a}	1.7±0.11^{a}	3.9±0.55^{a}
CS30	6.6±1.11^{b}	2.5±0.89^{b}	9.1±2.0^{b}
CS20+Fe&P	nd	nd	nd
CS30+Fe&P	nd	nd	nd

Mean values of triplicate groups, values are presented as mean ± SD. Values in the same column having different superscript letters are significantly different ($P < 0.05$).

nd: not detected.

Table 2-6. Total (+ and -) gossypol enantiomer accumulation in liver to several fish fed diets containing cottonseed meal.

Gossypol concentration	Parrot fish[1]	Flounder[2]	Rainbow trout[3]	Tilapia[4]
Dietary gossypol concentration (ug g^{-1})				
(+)-Enantiomer	783	934	1143	1140
(-)-Enantiomer	491	740	1125	1130
Total	1274	1674	2268	2270
Liver gossypol concentration (ug g wet $weight^{-1}$)				
(+)-Enantiomer	5.0	110	≒35	22.9
(-)-Enantiomer	2.3	40	≒15	9.4
Total	7.3	150	≒50	32.3

[1] In the present study

[2] Pham et al. (2007)

[3] Rinchard et al. (2003)

[4] Mbahinzireki et al. (2001)

2.4. Discussion

In this study, the crude protein (46 %, DM) and energy content (22 MJ/kg DM) of the experimental diets were formulated based on the protein and energy requirements of juvenile parrot fish as suggested by Kang et al. (1998). The results of the present study demonstrates that the mixture of cottonseed and soybean meal with lysine and methionine supplementation can replace dietary FM protein up to 20% without negative effects on weight gain or feed utilizations of juvenile (3-22 g) parrot fish. However, no reduction in growth performances or feed utilization were observed ongrowing (55-120 g) parrot fish fed up to 30% CS regardless of supplementation with iron and phytase compared to fish fed the FM based control diet. The same raw materials and experimental facilities were used for the two feeding trials. The reason for the increased replacement level (30%) compared to the result in experiment I (20%) might be attributed to the difference in fish size. More works are needed in order to gain insight into the effect of fish size on nutrient utilization.

The reduction in growth performance in experiment I may be attributed to the presence of antinutrional factors in cottonseed and/or soybean meal and/or available phosphorus level. In general, the phosphorus requirement of marine fish ranges from 0.5 to 1% of diet (Kim et al., 1998; Borlongan and Satoh, 2001; Roy and Lall, 2003; Oliva-Teles and Pimentel-Rodrigues, 2004). In a feeding study with Japanese sea bass the optimum dietary available phosphorus requirement for growth was 0.68% (Zhang et al., 2006). Mai et al. (2006) also reported that the optimum dietary available phosphorus level was 0.7% for maximum growth of yellow croaker. The dietary total phosphorus levels of the experimental diets in experiment I were calculated to range from 1.33 (CS50 diet) to 1.57% (CS30 diet) indicating that these levels would appear to meet the requirement for parrot fish. In plant protein sources, however, approximately 70% of

the total phosphorus is present as phytate phosphorus which cannot be absorbed and utilized by monogastric animals including fish (Lall, 1991). Therefore, the dietary available phosphorus levels in the plant protein-rich diets (CS30-50) in the experiment I may not be adequate to meet its requirement for optimal growth in juvenile parrot fish resulting in the impairment of growth performances due to the absence of intestinal phytase (Jackson et al., 1996).

A beneficial effect of phytase supplementation in diets on growth performance was not clearly demonstrated in the experiment II (compare diets CS20 and CS20+Fe&P, and CS30 and CS30+Fe&P). Yoo et al. (2005) reported that 30% dietary FM could be replaced by soybean meal with phytase supplementation in Korean rockfish. An improvement in growth performance of fish fed phytase supplemented diets has previously been reported (Rodehutscord and Pfeffer, 1995; Jackson et al., 1996; Papatryphon et al., 1999). In contrast, dietary supplementation of phytase did not improve growth performance in other studies (Lanari et al., 1998; Forster et al., 1999; Vielma et al., 2000; Sajjadi and Carter, 2004). The supplemental effect of phytase on growth performance in fish cannot simply be compared because it may differ depending on fish species, fish size, dietary phytase content or experimental conditions.

In the present study, significantly lower hematocrit and hemoglobin levels were observed in CS fed fish than in fish fed the FM based control diet. In previous studies, reduced hematocrit and hemoglobin were found in fish fed cottonseed meal containing diets (Dabrowski et al., 2000; Blom et al., 2001; Yildirim et al., 2003). This phenomenon could be explained by an adverse effect of gossypol on intestinal iron absorption (Braham and Bressani 1975). Skutches et al. (1974) demonstrated that dietary free gossypol can bind with iron resulting in a gossypol-iron complex in liver tissue. An increase in erythrocyte fragility has also been reported as a sign of gossypol toxicity in bovine (Brocas et al., 1997). However, none of these mechanisms has

been confirmed in fish because of some contradictory (Yildirim et al., 2004) and complicated (Barros et al., 2002) results in the observed hematological values.

The present study clearly demonstrated that dietary supplementation of CS significantly reduces plasma triacyglycerol and total cholesterol levels. The decrease in plasma cholesterol levels in fish fed diets containing plant protein has already been reported. Kaushik et al. (1995) reported that plasma cholesterol levels were reduced in rainbow trout fed soybean protein diets in comparison to those fed a FM diet. Likewise, Venou et al. (2006) reported that plasma cholesterol was decreased with inclusion of soybean meal in diets for gilthead seabream. In terrestrial animals, plant protein sources have generally been considered to have a hypocholesterolemic effect (de Schrijver, 1990), mainly due to the presence of the high levels of estrogenic isoflavones (Setchell and Cassidy, 1999). In experiment I, the dietary polyphenolic compound contents were 1466, 1538, 1595, 1707, 1833 and 1873 mg kg^{-1} diet for diets CS0, CS10, CS20, CS30, CS40 and CS50, respectively. CS contains various polyphenols, such as flavonoids, isoflavones, glycitein, genistein and daidzein, that may exert strong antioxidant effects (Andlauer et al., 1999; Fritz et al., 2003).

The toxicity of gossypol has been extensively studied and reported for humans and animals including fish (Colin-Negrete et al., 1996; Makinde et al., 1997; Lee, 2002). Gossypol toxicity depends on several factors including the isomeric form of gossypol [(+)- or (-)-enantimer], the amount consumed and the genetic variety of cottonseed. Channel catfish have been reported to tolerate up to 800 - 900 mg free gossypol/kg diet from either cottonseed gossypol or gossypol acetic acid with no adverse effects on weight gain or feed utilization (Yildirim et al., 2003). Mbahinzireki et al. (2001) reported that gossypol toxicity is the most important limiting factor for acceptance and utilization of cottonseed meal containing diets by Nile tilapia. It has been

well documented that gossypol can easily combine with lysine resulting in a deficiency of lysine (Robinson, 1991; Robinson and Li, 1994). In the present study, lysine and methionine were supplemented in the CS containing diets to meet their estimated requirements of fish. The growth rates observed in the current study suggest that gossypol levels up to 500 diet or 700 mg/kg diet are not a major factor in limiting the use of cottonseed meal in diets for juvenile (3-22 g) and ongrowing (55-120 g) parrot fish, respectively.

Iron as ferrous sulphate has been used to counteract the toxic effect of free gossypol for fish (Sealey et al., 1997; Barros et al., 2002; El-Saidy and Gaber, 2004). El-Saidy and Gaber (2004) reported that supplemental iron as ferrous sulphate at a 1:1 ratio of iron to free gossypol had no negative effect on dietary nutritional values. In terms of iron supplementation, there has been a report that a negative effect of increased susceptibility to *Edwardsiella ictaluri* infection was observed in channel catfish fed a high level of supplemental iron as ferrous sulphate (Sealey et al., 1997). No negative effect of the supplemental iron was observed in the present study. The gossypol enantiomer either (+)- or (-)- gossypol was not detected in liver of fish fed diets supplemented with iron. This result clearly supports the findings that supplemental iron in diets can reduce the toxicity of gossypol in fish by forming a complex compound in the intestinal tract which is excreted in the feces.

Liver is the major organ for gossypol accumulation in fish (Lee and Dabrowski, 2002). Yildirim et al. (2003) and a positive linear relationship between diet and liver gossypol content was found in channel catfish. In the present study, total and each (+)- or (-)-gossypol enantiomer concentration in the liver were slightly increased as the cottonseed meal inclusion increased in the diet except for the CS10 group. The total liver gossypol concentration of fish fed the diets containing cottonseed meal ranged from 2.9 to 7.3 ug/g wet tissues (from CS20 to CS50) except

for the CS10 group in which the gossypol was not detected. The gossypol concentrations in this study were lower than that observed in other studies. Roehm et al. (1967) reported a liver total gossypol content of 177 ug/g in rainbow trout fed purified diets supplemented with 250 ug/g gossypol for 12 months. Also, a total liver gossypol level of 207 ug/g was reported in channel catfish fed a semi-purified diet with 300 ug/g gossypol-acetic acid for 12 weeks (Yildirim et al., 2003). However, Robinson and Tiersch (1995) reported a total gossypol level of 54 ug/g dry liver in channel catfish fed a 400 ug/g free gossypol from a cottonseed based diet over 2 years. These results indicate that the liver gossypol accumulation in fish can significantly be influenced by the type of diets and/or the fish species tested.

In the present study, accumulation of total and (+ and -) gossypol enantiomer in liver of parrot fish (7.3 ug/g) is very low compared to other fish species (32 to 150 ug/g) even though the same source of gossypol and analytical methods were used (Table 2-6). The different patterns of accumulation may indicate that the uptake and/or metabolism of gossypol in parrot fish may differ from that of other fish species such as olive flounder (Pham et al., 2007), rainbow trout (Rinchard et al., 2003) and tilapia (Mbahinzireki et al., 2001). We are unable to explain the reason for the lower gossypol accumulation in liver of parrot fish than that of other fish species; however, it seems to be related to species-dependence differences. Further studies on a direct comparison are needed to investigate gossypol metabolism in tissues on other fish species.

In conclusion, the mixture of cottonseed and soybean meal with lysine and methionine supplementation can replace up to 20% FM protein in diets for juvenile (3-22 g) parrot fish. In ongrowing (55-120 g) parrot fish, FM protein can be replaced by up to 30% cottonseed and soybean meal protein although levels of FM replacement higher than 30% were not evaluated. A

benefit of dietary supplemental iron and phytase was not detected in the second feeding trial with the relatively low FM replacement levels. Our data suggest that up to approximately 30% FM protein can be replaced by an equal mixture of cottonseed and soybean meal with iron and phytase in the presence of lysine and methionine in practical diets for ongrowing parrot fish.

CHAPTER THREE

Taurine supplementation to alternative dietary proteins used in fish meal replacement enhances growth of juvenile parrot fish (*Oplegnsthus fasciatus*)

3.1. Introduction

Many studies on fish meal (FM) replacement in marine culture fish species have extensively conducted focusing on the use of plant protein sources due to increasing demand, limited supply and dramatic price increase of FM (Watanabe, 2002; Gatlin et al., 2007). Several studies have shown promising results in fish feed formulations for marine fish species and data have shown that approximately 20 to 40% FM protein can be replaced by plant protein sources in their diets (Chou et al., 2004; Hernandez et al., 2007; Lim and Lee, 2008, 2009). However, excessive replacement of FM by plant protein sources have generally resulted in inferior growth performance especially in carnivorous fish species and this is an obstacle in the development of low-cost fish feeds (Gaylord et al., 2006; Gatlin et al., 2007).

For this reason, many studies have been conducted to verify the limiting factors such as some amino acids (Li and Robinson, 1998; Cheng et al., 2003; Gaylord et al., 2007) and phosphorus (Albrektsen et al., 2006; Lim and Lee, 2008) on using plant protein sources in fish feeds. Data showed that fish growth rate is often reduced when compared to a FM based diets even when all the nutrient requirements are met in a plant based diets (Gaylord et al., 2006). From the results of these studies, it can be concluded that other nutrients that are not present in plant proteins may be supplied by FM and our attention has focused on taurine. Taurine has

been determined to be conditionally essential nutrient in plant protein based diets for some carnivorous fish species (Takeuchi, 2001). The efficacy of dietary taurine supplementation in improving growth performance has been reported in juvenile olive flounder (Kim et al., 2005a,b, 2007, 2008), red sea bream (Takagi et al., 2010), common dentex (Chatzifotis et al., 2008), cobia (Lunger et al., 2007) and yellowtail (Matsunari 2005; Takagi et al., 2008) fed a FM or plant protein based diets. Moreover, recent studies indicated that an totally plant protein based diet for rainbow trout was possible with a balanced combination of plant protein ingredients with supplementation of limiting nutrients, such as lysine, methionine, phosphate and taurine (Gaylod et al., 2007; Gaylord and Barrows, 2009; Lee et al., 2010).

Taurine, 2-amino ethanesulfonic acid, is a beta-amino sulfur amino acid that is found in high concentrations in most types of animal tissues. It is known to serve many important biological functions in mammals, including cell membrane stabilization (Pasantes-Morales et al., 1985), antioxidation (Nakamura et al., 1993), detoxification (Huxtable, 1992), osmoregulation (Thursion et al., 1980), neuromodulation (Bernardi, 1985) and brain and retinal development (Sturman, 1986). In mammals, the major pathway for taurine synthesize from methionine via cysteine involves the conversion of cysteine to cysteinesulfinate by cysteine dioxygenase, followed by its decarboxylation to hypotaurine by cysteinesulfinic acid decarboxylase (CSD) and then the oxidation of hypotaurine to taurine. Among these enzymes, the CSD has been demonstrated to be the rate limiting enzyme in taurine biosynthesis in many mammalian species (Jacobsen and Smith, 1968). In fish, activity of this enzyme varies in fish depending upon species and size, and the activities are lower than in mice and rats (Yokoyama et al., 2001). So, they suggested that fish may require dietary taurine as an essential nutrient similarly to cats and efforts to replace FM by plant proteins in their diets may necessitate dietary supplementation of taurine. Therefore, the experiment was conducted to determine whether taurine supplementation

to soybean meal-based diet can improve growth performance of juvenile parrot fish.

3.2. Materials and methods

3.2.1. Fish rearing

The feeding trial was conducted at the Marine and Environmental Research Institute, Jeju National University, Jeju, South Korea using juvenile parrot fish Oplegnathus fasciatus. The fish were from a genetically homogenous stock obtained by a private hatchery, Jeju Island, and adapted to a commercial diet (Suhyupfeed, co., Ltd, GyeongNam, South Korea). Experimental protocols followed the guidelines of the Animal Care and Use Committee of Jeju National University.

3.2.2. Diets and experimental design

The experimental diets (designated as FM, FM+T, SM, SM+TL, SM+TM and SM+TH, respectively) were formulated and fed to fish to determine whether taurine supplementation to plant protein-based diet would improve production performance of juvenile parrot fish. The FM diet was considered as the control diet. FM+T diet was prepared by adding 1% taurine. In SM diet, 30% FM protein in the FM diet was replaced by defatted soybean meal and supplemented with 0.05% (taurine content in FM diet), 1.0% (optimum level) or 2.0% (excessive level) taurine in SM diet, respectively (SM+TL, SM+TM or SM+TH). The soybean meal containing diets were supplemented with L-methionine, L-lysine and phosphorus to give levels comparative to the FM-based control diet. In the current study, the level (30%) of FM

replacement in the diets was determined from the earlier observation with juvenile parrot fish (3~20 g). FM protein was successfully replaced up to 20% by mixture of soybean meal and cottonseed meal (Lim and Lee, 2009). The dietary formulation, proximate composition, taurine content and amino acid composition are presented in Table 3-1 and 3-2.

All diets were prepared in the laboratory. Briefly, all the dry ingredients were minced and mixed in a feed mixer (NVM-16, Gyeonggido, South Korea) and then squid liver oil was added and mixed for 5 min. Distilled water was added to mixture (20-30 g/100 g of feed weight) and mixed until a pebble-like consistency was achieved. The mixture was then converted to pellets by a meat chopper machine (SMC-12, Kuposlice, Busan, South Korea). Pellets were dried at room temperature for 48 h, crushed into desirable particle sizes and stored in a freezer at –20 ^{o}C until used.

Table 3-1. Formulation and chemical analysis of the diets (% dry matter)

Ingredients	Experimental diets					
	FM	FM+T	SM	SM+TL	SM+TM	SM+TH
White fish meal	53.0	53.0	37.1	37.1	37.1	37.1
Soybean meal	-	-	23.4	23.4	23.4	23.4
Corn gluten meal	7.0	7.0	7.0	7.0	7.0	7.0
Wheat flour	7.0	7.0	7.0	7.0	7.0	7.0
Dextrin	15.0	15.0	6.7	6.7	6.7	6.7
Squid liver oil	11.0	11.0	11.8	11.8	11.8	11.8
Mineral mixture[a]	1.0	1.0	1.0	1.0	1.0	1.0
Vitamin mixture[b]	1.0	1.0	1.0	1.0	1.0	1.0
CMC	1.0	1.0	1.0	1.0	1.0	1.0
Lysine[c]	-	-	0.3	0.3	0.3	0.3
Methionine[d]	-	-	0.3	0.3	0.3	0.3
Phosphorus[e]	-	-	1.0	1.0	1.0	1.0
Taurine[f]	-	1.0	-	0.05	1.0	2.0
Cellulose	4.0	3.0	2.4	2.35	1.4	0.4
Chemical analysis (dry matter basis)						
Dry matter, %	8.3	8.1	8.5	8.7	8.4	8.6
Protein, %	45.8	46.7	46.4	46.0	46.8	47.2
Lipid, %	14.8	14.7	15.2	15.2	14.9	14.6
Ash, %	9.5	9.5	8.8	8.8	8.8	8.9
Crude fiber, %[g]	1.0	1.0	1.0	2.3	2.3	2.3
NFE, %[h]	28.9	28.1	28.6	27.7	27.2	27.0
Gross energy, MJ/kg[i]	21.6	21.7	21.9	21.6	21.6	21.5
Taurine, %	0.16	1.12	0.11	0.15	0.85	1.87

[a] Mineral premix (g kg^{-1} mixture) $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; CuCl, 0.2; $AlCl_3$. $6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[b] Vitamin premix (g kg^{-1} mixture) L-ascorbic acid, 121.2; DL-α tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-$_D$-pantothenate, 12.7; myo-inositol, 181.8; $_D$-biotin, 0.27; folic acid, 0.68; p-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003.

[c] L-lysine mono-hydrochloride, Sigma, USA

[d] L-methionine, Sigma, USA

[e] Monocalsium phosphate, Sigma, USA

[f] Taurine, Sigma, USA

[g] Fiber content was calculated based on fiber contents of white fish meal, soybean meal, corn gluten meal and wheat flour.

[h] Nitrogen-free extract (NFE) = 100-(% protein + % lipid + % ash + % fiber)

[i] Gross energy of experimental diets was calculated according to gross energy values 5.64 kcal/g protein, 4.11 kcal/g carbohydrate, and 9.44 kcal/g fat respectively (NRC, 1993).

Table 3-2. Amino acid composition of the diets (g $100g^{-1}$ diet, n=3)

Amino acid	FM	FM+T	SM	SM+TL	SM+TM	SM+TH
Lysine	3.11	3.11	3.09	3.02	3.07	3.06
Threonine	1.91	1.91	1.78	1.78	1.78	1.79
Methionine	1.48	1.49	1.44	1.54	1.50	1.38
Cystine	0.40	0.40	0.46	0.51	0.50	0.46
Isoleucine	1.67	1.68	1.69	1.66	1.64	1.63
Leucine	3.16	3.18	3.11	3.12	3.09	3.10
Valine	2.11	2.13	2.03	2.02	2.00	2.00
Arginine	2.76	2.71	2.74	2.74	2.73	2.76
Histidine	0.99	1.00	0.97	0.97	0.98	0.98
Phenylalanine	1.92	1.97	2.07	2.06	2.02	2.08
Serine	2.13	2.14	2.10	2.09	2.07	2.11
Alanine	2.81	2.84	2.41	2.41	2.42	2.42
Aspartic acid	4.10	4.11	4.18	4.18	4.16	4.18
Glutamic acid	6.22	6.13	6.52	6.51	6.53	6.50
Glycine	3.42	3.41	2.79	2.78	2.80	2.80
Proline	2.13	2.07	2.02	2.03	1.98	2.04

3.2.3. Feeding trials

After a week conditioning period, juvenile parrot fish with an initial body weight of 6.4±0.01g (mean±S.D.) were distributed to each tank as groups of 25 fish per tank and fed twice per day (8:30 h and 17:30 h) to apparent satiation, 7 days per week, for 12 weeks. The tanks were supplied with filtered seawater at a flow-rate of 2 L/min and aeration was used to maintain optimum dissolved oxygen level. Water temperature ranged from 24 to 28 °C according to the seasonal change. Weight and feed utilization of the fish were measured every 3 weeks to minimize stress of the fish.

3.2.4. Sample collection and analytical methods

At the beginning and the end of feeding trials, all the fishes were weighed. Weight gain, specific growth rate, feed efficiency, protein efficiency ratio and survival were calculated. Blood samples were obtained from the caudal vein of 6 fishes from each tank (18 fishes per dietary treatment) using heparinized syringe after anesthetization of the fish with tricaine methanesulfonate (MS-222) at a concentration of 100-200 mg/L. Hematocrit and hemoglobin were measured using microhematocrit technique (Brown, 1980) and CH 100 plus blood biochemical auto analyzer (SLIM, SEAC Inc, Florence, Italy). Analyses of crude protein, moisture and ash in the diets were performed by the standard procedures (AOAC International, 1997) and dietary lipid was determined according to the method described by Folch et al. (1957). Amino acid compositions in the diets were determined using a Sycom S-433D automatic amino acid analyzer (Sykam, Eresing, Germany). Hydrolysis of the flour samples was performed in 6N HCl at 110℃ for 24 h under nitrogen atmosphere. Identification and quantification of amino acids were achieved by comparing the retention times of the peaks with those of standards.

3.2.5. Palatability test

When the feeding trial was completed, palatability test was conducted under the same experimental tank and conditions. To measure palatability of experimental diets, the diets were weighed with 50g and the fish were fed their respective diets by hand for 5 min each morning for three days. The remaining feeds were then reweighed to determine the palatability. The test was conducted with 3 times and palatability was expressed as total consumed feed/10g fish.

3.2.6. Statistical analysis

All experimental diets were assigned by a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the differences in mean values were made with Duncan's multiple range test. Statistical significance was determined by setting the aggregate type I error at 5% ($P<0.05$) for each set of comparisons. Data are presented as mean±SD. Percentage data were arcsine transformed before statistical analysis.

3.3. Results

The experimental fish grew well during the feeding trial and mortality was lower than 5% in all the dietary groups. WG ranged from 1088 to 1139% and was significantly affected by the dietary treatment (Table 3-3). Fish fed the SM diet (replacement of 30% FM protein without supplemental taurine) had significantly lower WG than fish fed other diets. However, groups of fish fed the diets containing soybean meal with supplemental taurine (SM+TL, SM+TM and SM+TH) had significantly higher WG than fish fed the SM diet without taurine. WGs for groups of fish fed the FM, FM+T, SM+TM and SM+TH diets were similar whereas fish fed the SM+TL diet exhibited significantly lower one among the treatments. SGR followed the same trend as WG and fish fed the SM diet without supplemental taurine recorded the lowest SGR value. FE and PER also followed similar trends as WG and the lowest value was observed in fish fed the SM diet. Groups of fish fed the FM, FM+T, SM+TM and SM+TH diets were not significantly different on FE and PER whereas fish fed the SM+TL diet exhibited significantly lower than fish fed the FM+T or SM+TH diets.

The supplemental taurine in diets containing soybean meal significantly increased dietary palatability for juvenile parrot fish (Fig. 3-1). The lowest palatability activity was observed in fish fed the SM diet without supplemental taurine and groups of fish fed the SM+TM and SM+TH diets were significantly higher than fish fed the SM diet without supplemental taurine.

Blood parameters were significantly affected by the dietary treatment (Table 3-4). The lowest hematocrit value was observed in fish fed the SM diet without supplemental taurine and the value was significantly lower than fish fed the FM+T diet. However, no significant differences were observed aming groups of fish fed the FM, FM+T, SM+TM and SM+TL diets. Hemoglobin values followed the same trend as hematocrit.

Table 3-3. Growth performance of juvenile parrot fish in the feeding trials fed different experimental diets for 12 weeks.

Diets	FM	FM+T	SM	SM+TL	SM+TM	SM+TH
Dietary taurine, %	0.16	1.12	0.11	0.15	0.85	1.87
WG (%)[a]	1132±5.5[c]	1139±14.5[c]	1034±12.9[a]	1088±28.7[b]	1131±14.0[c]	1128±18.1[c]
SGR (%)[b]	3.03±0.01[c]	3.03±0.01[c]	2.93±0.01[a]	2.98±0.03[b]	3.02±0.01[c]	3.02±0.02[c]
FE[c]	0.91±0.01[bc]	0.93±0.02[c]	0.85±0.01[a]	0.88±0.03[ab]	0.90±0.01[bc]	0.91±0.01[c]
PER[d]	2.00±0.03[bc]	2.06±0.05[c]	1.88±0.02[a]	1.94±0.06[ab]	1.99±0.01[bc]	2.02±0.01[c]
Survival (%)	100±0.0	100±0.0	98.3±2.9	94.7±4.6	97.3±2.3	97.3±2.3

Means of triplicate groups, values are presented as mean ± SD. Values in the same row having different superscript letters are significantly different ($P < 0.05$).

[a] Weight gain (%) = 100 x (final mean body weight - initial mean body weight) x initial mean body weight^{-1}

[b] Specific growth rate (%) = [(loge final body weight - loge initial body weight) x days^{-1}] x 100

[c] Feed efficiency = wet weight gain x dry feed fed^{-1}

[d] Protein efficiency ratio = wet weight gain x total protein given^{-1}

Table 3-4. Blood parameters of juvenile parrot fish in the feeding trials fed different experimental diets for 12 weeks.

Diets	FM	FM+T	SM	SM+TL	SM+TM	SM+TH
Dietary taurine, %	0.16	1.12	0.11	0.15	0.85	1.87
Hematocrit (%)	38.7±5.2^{c}	43.2±2.1^{c}	37.0±2.0^{a}	39.3±1.8^{b}	38.4±1.4^{c}	39.7±2.6^{c}
Hemoglobin (g/dL)	7.8±1.0^{c}	8.9±0.2^{c}	7.3±0.7^{a}	8.2±0.2^{b}	7.9±0.4^{c}	8.1±0.4^{c}

Mean values of triplicate groups, values are presented as mean ± SD. Values in the same row having different superscript letters are significantly different ($P < 0.05$).

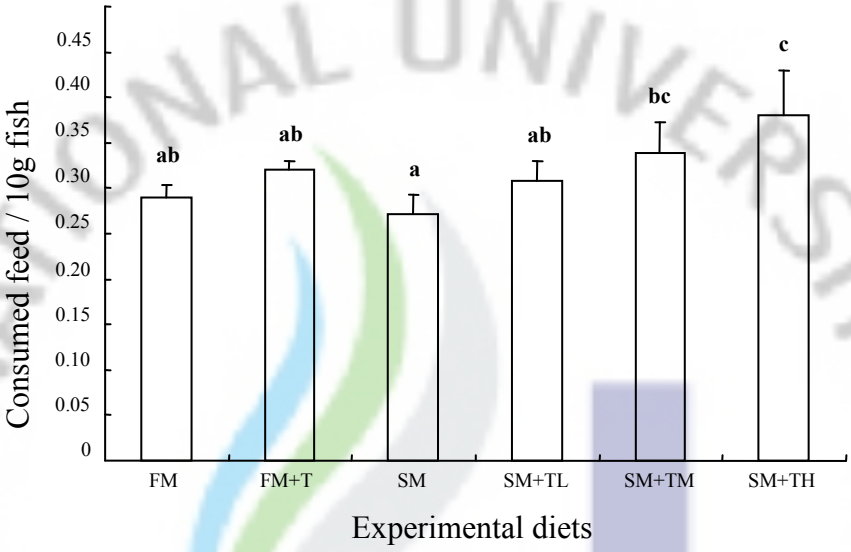

Fig. 3-1. Palatability activity for juvenile parrot fish fed the experimental diets for 5 min. Values represent mean±standard errors of each of triplicate groups. Bars with different letters are significantly different ($P<0.05$).

3.4. Discussion

Based on the results of previous studies on taurine in fish, carnivorous fish species may be unable to biosynthesize taurine from cysteine. For example, activity of CSD, rate limiting enzyme in taurine biosynthesis, is not present in the yellowtail, bluefin tuna and skipjack tuna (Yokoyama et al., 2001). Dietary taurine supplementation has improved growth of several carnivorous fish species, such as olive flounder (Kim et al., 2005a,b), red seabream (Matsunari et al., 2008), cobia (Lunger et al., 2007), yellowtail (Matsunari et al., 2005), common dentex (Chatzifotis et al., 2008) and rainbow trout (Gaylord et al., 2006). This phenomenon could be explained by their feeds in nature that contains high level of taurine. In wild environment, the fish species can be satisfied with required taurine for growth or normal physiological functions by their feeds, thus may be less able to synthesize taurine. Herbivorous fish species may be more capable of taurine biosynthesis because of lack of taurine in their feeds (Lunger et al., 2007). Against this background, the current study was undertaken to confirm necessity of taurine supplementation for maximum growth of juvenile parrot fish fed a fish meal or plant based diets.

In the current study, taurine supplementation positively affected growth and feed utilization of juvenile parrot fish fed fish meal or soybean meal based diets (30% replacement of fish meal protein). These findings indicate that taurine is essential for maximum growth of juvenile parrot fish, even though FM was used as the main protein source in their diet. Efficacy of taurine supplementation to a fish meal based diet on growth and feed efficiency has been reported in several fish species, such as European eel (Sakaguchi et al., 1988), olive flounder (Kim et al., 2005a,b, 2007, 2008), yellowtail (Matsunari et al., 2005) and European seabass (Martinez et al., 2004). In this study, taurine supplementation (1% of dry diet) to the FM-based diet (53% white

FM) significantly increased growth performance of juvenile parrot fish (6-40g) over the 6 weeks period, but over 12 weeks, growth performance of juvenile parrot fish (40-80g) was not significantly related to taurine supplementation. These results suggest that taurine has an important role on growth performance during the early juvenile stage in parrot fish. A similar observation was made for juvenile olive flounder (Kim et al., 2003) and juvenile yellowtail (Matsunari et al., 2005). Kim et al. (2005b) reported that the early stage juvenile olive flounder (initial size 0.3g) require taurine in their diets but this requirement is reduced for the larger juvenile fish (initial size 3.7g). Kim et al. (2003) also suggested that taurine has an important role only during the juvenile period (initial size 0.4g) of olive flounder, not the fingerling period (initial size 14.7g).

Recent studies have demonstrated that several fish species require dietary taurine as an essential nutrient especially in reduced fish meal diets or all-plant diets. It was reported that growth and feed utilization of red seabream (Takagi et al., 2006, 2010), cobia (Lunger et al., 2007) and rainbow trout (Gaylord et al., 2006, 2007) fed low levels fish meal diets are improved by dietary taurine supplementation. In the present study, tauine supplementation to the SM diet, a 30% substitution level of fish meal by soybean meal, improved weight gain, specific growth rate, feed efficiency, and protein efficiency ratio of juvenile parrot fish. Moreover, growth performance and feed utilization of fish fed the SM diets with ≥0.85% taurine were not significantly different compared to that of fish fed the FM diet. This finding indicates that a level of dietary taurine about 1% is sufficient to maintain growth and feed efficiency of juvenile parrot fish (6-80g).

Taurine has been reported to have a stimulatory effect on palatability in Artic charr and greyling (Doving et al., 1980), red sea bream (Fuke et al., 1981), rainbow trout (Hara et al., 1984), European glass eel (Sola and Tosi, 1993), and olive flounder (Kim et al., 2005b). In the present study, the palatability activity was also increased with taurine supplementation to the SM diet. Therefore, the increased growth performance and feed utilization of fish fed diets containing soybean meal with supplemental taurine can be attributed to the increased feed intake and improved feed efficiency. In other words, taurine supplementation to diets for juvenile parrot fish can make SM diets more palatable, thus increasing feed intake and subsequently weight gain.

It is known that hematocrit and hemoglobin values are usually used as indicators of general health in fish (Tort et al., 1996). Many researchers have found that hematocrits and hemoglobin varies according to the deficiency of essential nutrients, environmental conditions, growth status or anti-nutritional factors (Garrido et al., 1990; Lim and Lee, 2009). In this study, the lowest hematocrit value was observed in fish fed the SM diet without supplemental taurine and the value was significantly lower than fish fed the FM diet with supplemental taurine. However, no significant differences were observed in fish fed the SM diets with supplemental taurine. Therefore, the reason for the lower blood hematocrit and hemoglobin in fish fed the SM diet might be mainly due to the deficiency of dietary taurine. A similar observation was reported in yellowtail fed a non-fish meal diet with supplemental taurine (4.5%) for 39 weeks (Takagi et al., 2008). The results of the current study indicate that taurine supplementation may be necessary for juvenile parrot fish fed low levels of fish meal in diets based on plant proteins.

In conclusion, the supplementation of taurine to a soybean meal based diet promotes the feed intake and growth performance and 30% fish meal can be replaced by soybean meal with

approximately 1% taurine supplementation in the presence of lysine, methionine and phosphorus in practical diets for juvenile parrot fish (6-80g). The result of the present study is significant because it is the first dietary formulation with taurine for parrot fish with its optimal level. Further research is needed to determine a requirement or essentiality of taurine in other carnivorous fish species and to clarify the physiological role of taurine in fish.

CHAPTER FOUR

Supplemental taurine increase dietary inclusion of alternative proteins used in fish meal replacement in parrot fish (*Oplegnsthus fasciatus*)

4.1. Introduction

In aquaculture production, fish meal is typically regarded as the main protein source in diets for carnivorous fish due to its high level of protein, excellent amino acid profile which provides adequate levels of all essential amino acids, low carbohydrate level, high digestibility, and few antinutritional factors (Zhou et al., 2004). However, due to the stagnant supply of fish meal, prices will inevitably increase with demand (FAO, 2004; Lunger et al., 2007). This has amplified the need to investigate alternative protein sources. The use of plant proteins in diets for carnivorous species creates a challenge since they typically require higher levels of protein in their diets and plant proteins are less palatable. Nevertheless, several studies have shown promising results using plant-based protein sources in aquafeed formulations (Gomes et al., 1995; McGoogan and Gatlin, 1997; Chou et al., 2004). Plant protein sources that have received the most interest are soybean meal and corn gluten meal due to their good amino acid profiles except for methionine, which is limiting in soybean meal (El-Sayed, 1999), and lysine, which is limiting in corn gluten mean (Pereira and Oliva-Teles, 2003).

Soybean meal (SM) has been the most frequently studied dietary ingredient as a FM replacer in diets for many fish species because of its high protein content, relatively well-balanced amino acid profiles, reasonable price and steady supply. (Storebakken et al., 2000).

The value of SM as a substitute for FM in formulated diets has been investigated for many fish species, such as Atlantic salmon (Refstie et al., 2001), Asian sea bass (Boonyaratpalrin et al., 1998), channel catfish (Bai and Gatlin, 1994), rainbow trout (Cho et al., 1974), grass carp (Dabrowski and Kosak, 1979) and common carp (Viola et al., 1983). Kikuchi (1999) reported that about 45% of FM protein could be replaced with SM in combination with other protein sources in the diet of olive flounder. However, the use of SM in fish feed is still limited because of the presence of some antinutritional factors, such as protease inhibitors, phytates, lectins, saponins, non-starch polysaccharide and high fiber content (Refstie et al., 1999; Storebakken et al., 2000; Hendricks, 2002). In addition, the deficiency of some essential amino acids in SM, such as methionine and lysine also reduces the inclusion level of this material in fish feeds (NRC, 1993; Krogdahl, 1995).

One major obstacle in using plant protein sources is the presence of phytic acid (NRC, 1993). Phytic acid (myo-inositol hexakisphosphate) is the major compound for phosphorus storage (over 70%) in the plant seeds and cannot be digested and absorbed by monogastric animals including fish (Barual et al., 2004). Many fish nutritionists have tried to supplement phosphorus itself as phosphate to compensate unavailable phosphorus in the plant seeds and/or phytase, an enzyme, to liberate free phosphorus from phytic acid. In Atlantic, cod phosphorus supplementation in plant protein based diets could replace 50% dietary FM without growth impairment (Albrektsen et al., 2006). Also, soybean meal was successfully replaced by cottonseed meal with supplementation of phosphorus as dicalciumphosphate in channel catfish diets (Robinson and Brent, 1989; Robinson and Tiersch, 1995).

Taurine is not considered to be an essential amino acid because it can be synthesized by fish. As in mammals, Yokoyama et al. (1997) demonstrated that rainbow trout synthesized

taurine from cysteine. In the mammalian system, taurine is synthesized through many enzymatic reactions; but the enzyme L-cysteinesulphinate decarboxylase appears to be rate-limiting (Jacobsen and Smith, 1968). Activity of this enzyme varies in fish depending upon species and size. For example, in the yellowtail, as well in bluefin and skipjack tunas, L-cysteinesulphinate decarboxylase activity is not present, whereas in Japanese flounder it expresses only low activity (Yokoyama et al., 2001).

Taurine is typically found in relatively high concentrations in fish meal and animal by-products but is almost non-existent in plant meals. Even when all essential amino acid requirements are met in plant-based diets for carnivorous fish, growth is often reduced when compared to fish meal-based diets (Gaylord et al., 2006). Therefore, taurine supplementation may be required for plant-based diets and indeed, dietary taurine additions improve weight gain and feed efficiency in olive flounder (Park et al., 2002; Kim et al., 2005a), as well as in rainbow trout (Gaylord et al., 2006). Based on this information, the current studies were undertaken to examine whether higher levels of fish meal could be replaced in parrot fish diets utilizing soybean meal protein with supplementation of taurine.

4.2. Materials and methods

4.2.1. Fish rearing

The feeding trial were conducted at the Marine and Environmental Research Institute, Jeju National University, Jeju, South Korea using juvenile parrot fish *Oplegnathus fasciatus*. The fish were from a genetically homogenous stock obtained by a private hatchery, Jeju Island, and adapted to a commercial diet (Suhyupfeed, co., Ltd, GyeongNam, South Korea). Experimental protocols followed the guidelines of the Animal Care and Use Committee of Jeju National University.

4.2.2. Diets and experimental design

The experimental diets were formulated and fed to fish to determine whether taurine supplementation to plant protein-based diet would improve production performance of juvenile parrot fish. Seven experimental diets were formulated to replace FM protein by soybean meal at 0, 20, 30, or 40% (designated as FM0, SM20, SM30 or SM40, respectively). Three additional diets were manufactured by adding taurine (1.0 g/100 g dry diet) at each fish meal replacement diets, 20, 30 or 40% (designated as SM20-T, SM30-T or SM40-T, respectively). Each feed was fed in triplicates. The soybean meal containing diets were supplemented with L-methionine, L-lysine and phosphorus to give levels comparative to the FM-based control diet. In the current study, the level of FM replacement in the diets was determined from the earlier observation with juvenile parrot fish (3~20 g). FM protein was successfully replaced up to 20% by mixture of soybean meal and cottonseed meal (Lim and Lee, 2009). The dietary formulation, proximate composition and taurine content and dietary amino acid composition are presented in Table 4-1.

All diets were prepared in the laboratory. Briefly, all the dry ingredients were minced and

mixed in a feed mixer (NVM-16, Gyeonggido, South Korea) and then squid liver oil was added and mixed for 5 min. Distilled water was added to mixture (20-30 g/100 g of feed weight) and mixed until a pebble-like consistency was achieved. The mixture was then converted to pellets by a meat chopper machine (SMC-12, Kuposlice, Busan, South Korea). Pellets were dried at room temperature for 48 h, crushed into desirable particle sizes and stored in a freezer at –20 ^{o}C until used.

Table 4-1. Formulation and chemical analysis of the diets (% dry matter)

Ingredients (%)	FM	SM20	SM20-T	SM30	SM30-T	SM40	SM40-T
Fish meal	48.0	38.4	38.4	33.6	33.6	28.8	28.8
Soybean meal	0.0	14.0	14.0	21.1	21.1	28.2	28.2
Corn gluten meal	9.0	9.0	9.0	9.0	9.0	9.0	9.0
Wheat flour	13.0	13.0	13.0	13.0	13.0	13.0	13.0
Dextrin	12.0	8.0	8.0	5.3	5.3	3.0	3.0
Vitamin mix [a]	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Mineral mix [b]	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Squid liver oil	11.0	11.5	11.5	11.8	11.8	12.0	12.0
CMC	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Lysine [c]	0.0	0.2	0.2	0.3	0.3	0.4	0.4
Methionine [d]	0.0	0.2	0.2	0.3	0.3	0.4	0.4
Phosphorus [e]	0.0	0.8	0.8	1.0	1.0	1.2	1.2
Cellulose	4.0	2.3	1.3	1.6	0.6	1.0	0.0
Taurine [f]	0.0	0.0	1.0	0.0	1.0	0.0	1.0

[a] Mineral premix (g kg^{-1} mixture) $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; CuCl, 0.2; $AlCl_3$. $6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[b] Vitamin premix (g kg^{-1} mixture) L-ascorbic acid, 121.2; DL-tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-$_D$-pantothenate, 12.7; myo-inositol, 181.8; $_D$-biotin, 0.27; folic acid, 0.68; p-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003.

[c] L-lysine mono-hydrochloride, Sigma, USA

[d] L-methionine, Sigma, USA

[e] Monocalsium phosphate, Sigma, USA

[f] Taurine, Sigma, USA

4.2.3. Feeding trials

After a week conditioning period, juvenile parrot fish with an initial body weight of 30±0.1g (mean±S.D.) were distributed to each tank as groups of 15 fish per tank and fed twice per day (8:30 h and 17:30 h) to apparent satiation, 7 days per week, for 8 weeks. The tanks were supplied with filtered seawater at a flow-rate of 2 L/min and aeration was used to maintain optimum dissolved oxygen level. Water temperature ranged from 20 to 25 ^{o}C according to the seasonal change. Weight and feed utilization of the fish were measured every 4 weeks to minimize stress of the fish.

4.2.4. Sample collection and analytical methods

At the beginning and end of feeding trials, all the fishes were weighed. Weight gain, specific growth rate, feed efficiency, protein efficiency ratio and survival were calculated. Blood samples were obtained from the caudal vein of 6 fishes from each tank (18 fishes per dietary treatment) using heparinized syringe after anesthetization of the fish with tricaine methanesulfonate (MS-222) at a concentration of 100-200 mg/L. Hematocrit and hemoglobin were measured using microhematocrit technique (Brown, 1980) and CH 100 plus blood biochemical auto analyzer (SLIM, SEAC Inc, Florence, Italy), respectively. Analyses of crude protein, moisture and ash in the diets were performed by the standard procedures (AOAC International, 1997) and dietary lipid was determined according to the method described by Folch et al. (1957). Amino acid compositions in the diets were determined using a Sycom S-433D automatic amino acid analyzer (Sykam, Eresing, Germany). Hydrolysis of the flour samples was performed in 6N HCl at 110℃ for 24 h under nitrogen atmosphere. Identification

and quantification of amino acids were achieved by comparing the retention times of the peaks with those of standards.

4.2.5. Palatability test

When the feeding trial was completed, palatability test was conducted under the same experimental tank and conditions. To measure palatability of experimental diets, the diets were weighed with 50g and the fish were fed their respective diets by hand for 5 min each morning for three days. And then, remaining feeds were reweighed to determine the palatability activity. The test was conducted with 3 times and palatability activity was expressed as total consumed feed/10g fish.

4.2.6. Statistical analysis

All experimental diets were assigned by a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the differences in mean values were made with Duncan's multiple range test. Statistical significance was determined by setting the aggregate type I error at 5% ($P<0.05$) for each set of comparisons. Data are presented as mean±SD. Percentage data were arcsine transformed before statistical analysis.

4.3. Results

The experimental fish grew well during the feeding trial and mortality was lower than 5% in all the dietary groups (Fig. 4-1). WG ranged from 187 to 209% and was significantly affected by the dietary treatment (Fig. 4-2). Parrot fish that were fed the 20 and 30% soybean meal based diets supplemented with taurine had significantly higher weight gains than the 40% soybean meal based diets without taurine supplementation. Fish fed SM40 diet (40% soybean meal based protein without taurine) had significantly poorer growth than all other fish. Specific growth rates (SGR) ranged from 2.03 to 2.17 and also were significantly impacted by diet (Fig. 4-3). SGRs for parrot fish fed the control diet (FM) and the 20, 30 and 40% soybean meal based diets with taurine were similar whereas fish fed the 20%, 30% and 40% soybean meal based diets without supplemental taurine exhibited lower SGR values. Fish fed the 40% soybean meal based protein diet without taurine recorded the lowest SGRs in the feeding trial. Feed efficiency (FE) values ranged from 0.82 to 0.94 (Fig. 4-4) and followed a similar trend as weight gain. Parrot fish fed diets supplemented with taurine had significantly higher FE values than that of without supplemental taurine. FE values for the control diet and the 30% soybean meal protein diet with taurine were the same, but fish fed the 40% soybean meal based diet without taurine had significantly lower FE values. Protein efficiency ratio ranged from 1.79 to 2.05 and followed a similar trend as FE values (Fig. 4-5).

The supplemental taurine in diets containing soybean meal significantly increased dietary palatability for juvenile parrot fish (Fig. 4-6). The lowest palatability activity was observed in groups of fish fed the SM diet without supplemental taurine (SM20, SM30 and SM40). Groups of fish fed the SM diet with supplemental taurine (SM20-T and SM40-T) had significantly higher than fish fed the SM diet without supplemental taurine.

Blood parameters were not significantly affected by the dietary treatment (Table 4-2). No significant differences were observed in groups of fish fed the experimental diets.

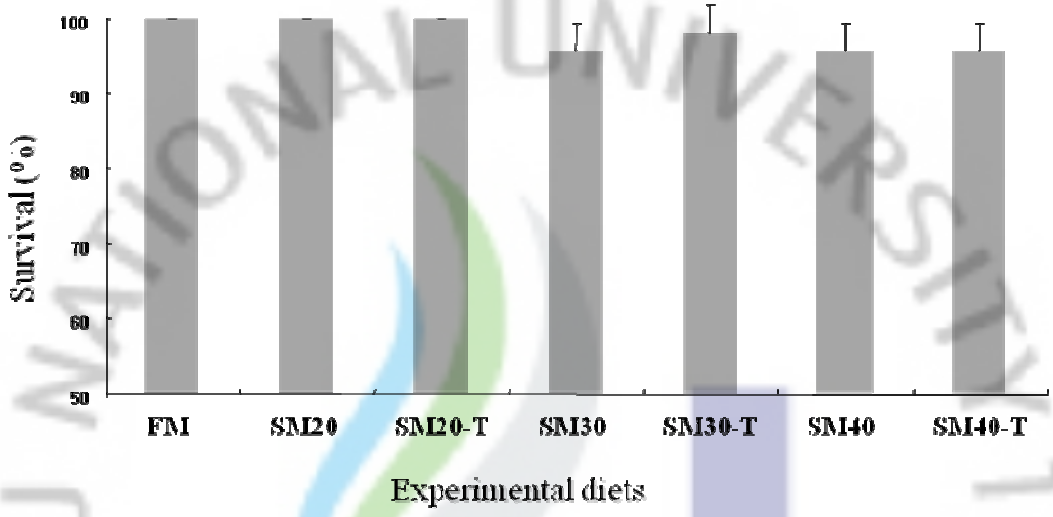

Fig. 4-1. Survival of juvenile parrot fish in the feeding trials fed different experimental diets for 8 weeks.

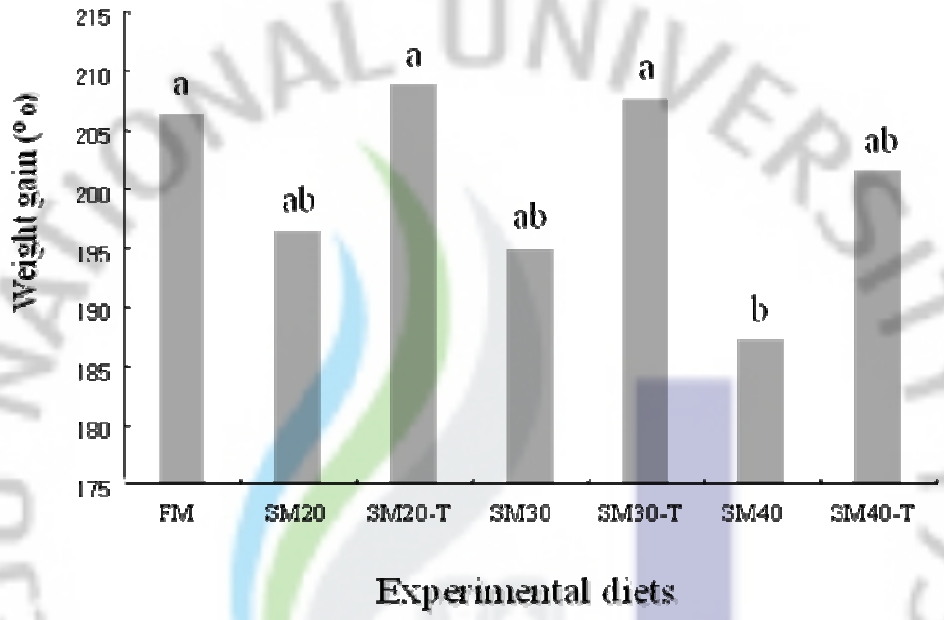

Fig. 4-2. Weight gain (%) of juvenile parrot fish in the feeding trials fed different experimental diets for 8 weeks.

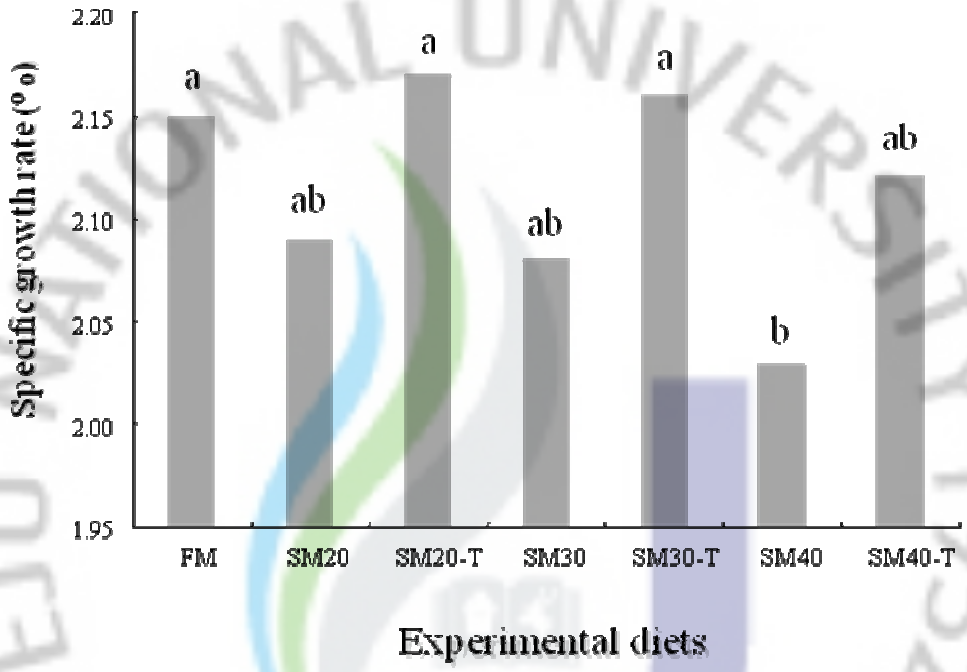

Fig. 4-3. Specific growth weight of juvenile parrot fish in the feeding trials fed different experimental diets for 8 weeks.

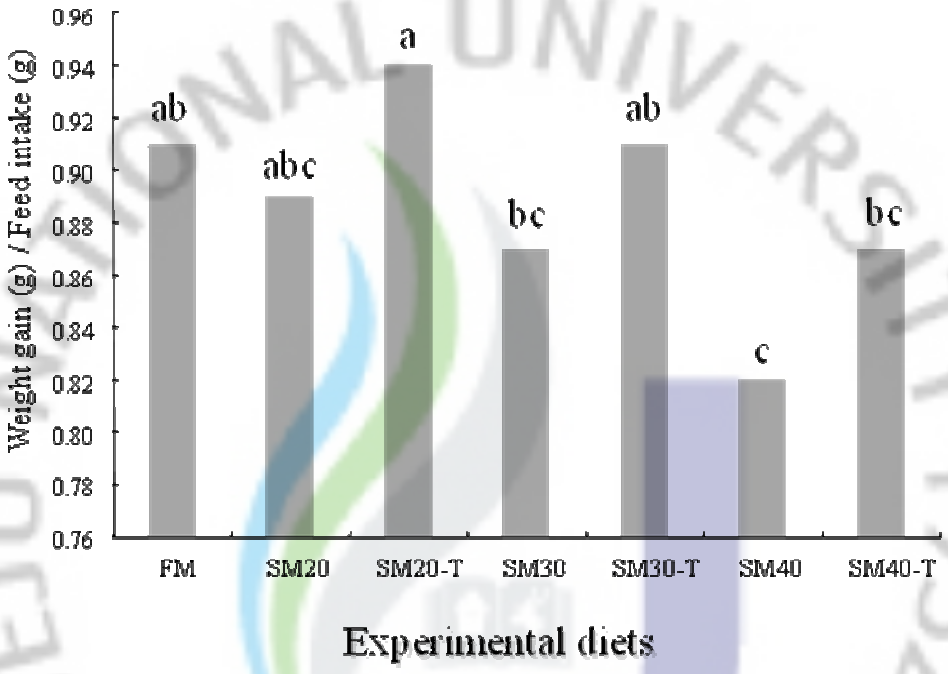

Fig. 4-4. Feed efficiency of juvenile parrot fish in the feeding trials fed different experimental diets for 8 weeks.

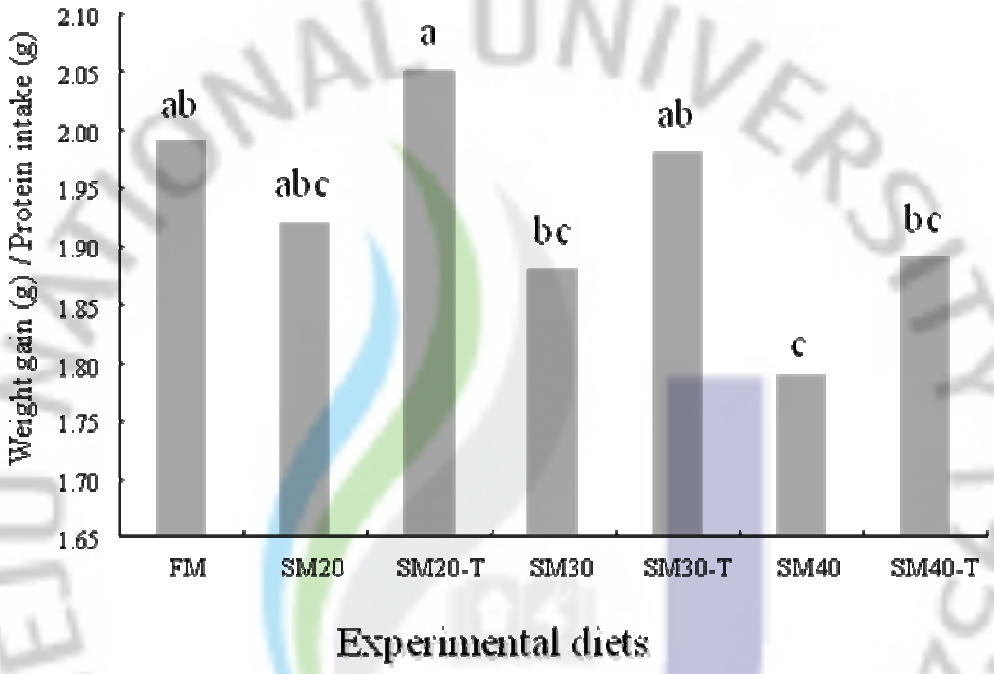

Fig. 4-5. Protein efficiency ratio of juvenile parrot fish in the feeding trials fed different experimental diets for 8 weeks.

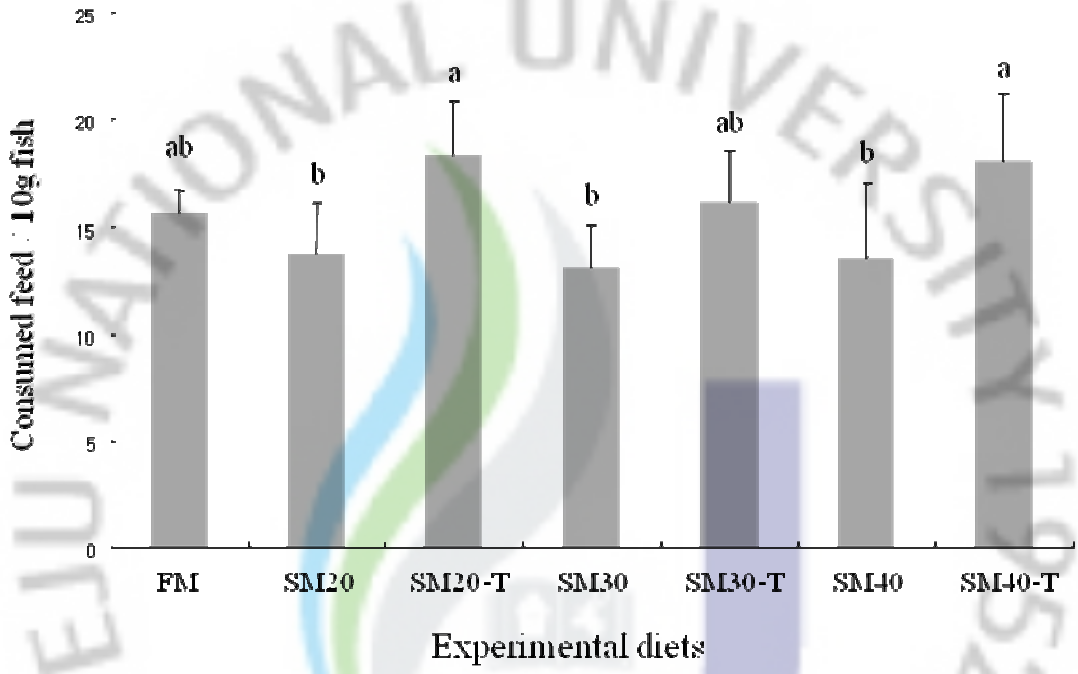

Fig. 4-6. Palatability activity for juvenile parrot fish fed the experimental diets for 5 min. Values represent mean±standard errors of each of triplicate groups. Bars with different letters are significantly different ($P<0.05$).

Table 4-2. Blood parameters of juvenile parrot fish in the feeding trials fed different experimental diets for 8 weeks.

Diets	FM	SM20	SM20-T	SM30	SM30-T	SM40	SM40-T
Hematocrit (%)	32.3±0.7	32.4 2.4	33.4±4.0	32.0±2.5	31.4±2.3	29.7±5.3	32.9±1.0
Hemoglobin (g/dL)	6.5±0.4	6.5 0.1	6.3±1.0^{c}	5.8±0.6	6.8±0.5	6.0±0.2	6.7±0.5

Mean values of triplicate groups, values are presented as mean ± SD. Values in the same row having different superscript letters are significantly different ($P < 0.05$).

4.4. Discussion

Carnivorous fish in the wild consume relatively large quantities of taurine since it is highly abundant in animal tissues, but this does not apply when diets contain large amounts of plant protein sources in our feeding trials we added taurine at 1.0 g/ 100 g dry weight to diets. Fish fed diets supplemented with taurine gained significantly more weight and better feed efficiency than fish fed the control diet. Growth rates and feed efficiency also have been improved with taurine supplementation in species such as olive flounder (Park et al., 2002; Kim et al., 2003, 2005a,b), European seabass (Martinez et al., 2004), yellowtail (Matsunari et al., 2005) and rainbow trout (Gaylord et al., 2006). In our study, weight gain, specific growth rate, feed efficiency and protein efficiency ratio were also improved with taurine supplementation, but all measurements tended to decrease with increasing level of dietary fish meal replacement. The same trend was observed in juvenile parrot fish when the mixture of soybean meal and cottonseed meal protein was replaced at identical levels without taurine (Lim and Lee, 2009). In our previous study feed intake of fish fed diets containing high levels of plant protein was low. The diet containing 30 or 40% of the plant protein source in Lim and Lee (2009) was repeatedly spit out by the parrot fish, whereas the same diet with taurine addition in the present trial was readily and eagerly consumed by the parrot fish, indicating no palatability issues. It has been reported that taurine can act as a feed attractant and European seabass fry were observed to preferentially consume a diet supplemented with 0.2% taurine (Martinez et al., 2004).

Evidence from the present studies indicates that taurine is conditionally indispensable when parrot fish are fed diets containing high levels of plant-based protein sources. Martinez et al. (2004) reported that seabass may require dietary taurine supplementation under certain feeding practices as well. Rainbow trout fed a plant-based diet required taurine supplemented at

5 g/kg dry diet in order to keep up with the growth of fish fed a fish meal-based control diet (Gaylord et al., 2006). Kim et al. (2003) reported that juvenile olive flounder required taurine supplementation, whereas fingerling olive flounder did not. Optimal levels of taurine supplementation were suggested to be 15 mg/g (Kim et al., 2005b) or 15–20 mg/g (Park et al., 2001). These results when taken together, indicate that taurine supplementation is necessary for carnivorous fish species when fed diets with alternate protein sources. This could be particularly true for marine species since taurine plays a critical role in osmoregulation and typically comprises more than 50% of the free amino acid pool (Lombardini et al., 1979). Fish raised in sea water may have a greater demand for dietary taurine than fish held in fresh water and a fish's ability to convert cysteine to taurine may be based on their environmental salinity requirements (Gaylord et al., 2006). Therefore, the osmotic stabilization provided by taurine may be related to its effects on growth and the fact that supplementation to diets improves growth in numerous species (Kim et al., 2003). Some species may be unable or poor at synthesizing taurine *de novo* from cysteine. This result may be due to the activity level of L-cysteinesulphinate decarboxylase, which in turn might be influenced by the natural feeding habits of a particular species or previous feeding history (Gaylord et al., 2006). Carnivorous fish, therefore, may be less able to synthesize taurine due to their naturally high intake while herbivorous/omnivorous fish may be more capable of such synthesis due to the paucity of taurine in their diets. Species with rapid growth rates, such as cobia, may also experience an increased demand on the *de novo* synthesis of taurine which cannot be met, especially when fish meal is replaced in diets by plant protein sources devoid of taurine. When a non-essential amino acid, such as taurine, is added to diets, it may be possible to conserve essential amino acids as well (Cowey, 1994), which could lead to improved growth rates. In our study fish were fed diets containing 20, 30 and 40% soybean meal with methionine and lysine or methionine, lysine and taurine. The diets supplemented with methionine and lysine alone resulted in inferior weight

gains compared to other diets containing three of them. A reason for these results could be that when taurine was supplemented alone, its presence allowed parrot fish to conserve the essential amino acids (methionine) that were present in the unsupplemented diets thus improving growth rates.

Taurine has been reported to have a stimulatory effect on palatability in Artic charr and greyling (Doving et al., 1980), red sea bream (Fuke et al., 1981), rainbow trout (Hara et al., 1984), European glass eel (Sola and Tosi, 1993), and olive flounder (Kim et al., 2005b). In our study, the palatability activity was also increased with taurine supplementation to the soybean meal diet. Therefore, the increased growth performance and feed utilization of fish fed diets containing soybean meal with supplemental taurine can be attributed to the increased feed intake and improved feed efficiency. That is, dietary taurine supplementation for ongrowing parrot fish can make soybean meal diets more palatable and available, thus increasing feed intake and subsequently weight gain.

Due to the wide range of biological impacts associated with taurine, including anticonvulsant activity, muscle membrane stabilization, bile salt synthesis, cell proliferation and viability and antioxidant activities (Huxtable, 1992), the reasons for the improved growth rates observed in the present study with the addition of taurine are purely speculation. This particular area of research is very limited and results such as these certainly warrant future investigations. It is obvious from the results of the present studies that taurine supplementation does have a significant impact on growth and feed efficiency of ongrowing parrot fish when they are fed diets containing high levels of plant protein sources as replacement for fish meal. These findings could dramatically change the amount and types of alternate proteins that can be effectively incorporated into diets for parrot fish and decrease the industries reliance on fish meal supplies.

The results from this study also magnify the importance of quantitative amino acid requirements for parrot fish, many of which are presently undetermined.

CHAPTER FIVE

Taurine is an essential nutrient in diet for parrot fish (*Oplegnsthus fasciatus*)

5.1. Introduction

Dietary replacement of fish meal (FM) has been an important issue in aquaculture industry due to a limited supply of FM and its dramatic price increase in recent years (FAO, 2004). Feed costs account for over 50% total production costs in most marine fish species, because of the use of the expensive FM with a large dietary proportion (Coyle et al., 2004). FM has been a major ingredient in fish diets because of its high protein quality and palatability. Substituting less expensive protein sources for high-price FM in fish feeds is one way to lower production costs (Lee et al., 2001). For this reason, many studies have been conducted to replace or reduce its inclusion in fish diets by various cheaper alternative animal and vegetable protein sources; however, each candidate has characteristics that make it inferior in some respect to high-quality FM (Hardy, 1996).

Taurine has been implicated to play roles in bile salt synthesis, osmoregulation, modulation of neurotransmitters and hormone release, and antioxidation in mammals (Huxtable, 1992). It is also present in various tissues of marine fishes at considerably high concentrations (Ozawa et al., 1984; Sakaguchi et al., 1988). Although taurine is a well known constituent in fish, there is little information available on its applications to aquaculture.

Recent investigations have indicated that taurine is an essential element for some kinds of larval and juvenile marine finfish (Takeuchi et al., 2001; Yokoyama et al., 2001). For example, supplementation of taurine in the diet improves the growth of the juvenile Japanese flounder (Park et al., 2002; Kim et al., 2003) and taurine enrichment of rotifers is effective to improve the growth and survival in red seabream larvae (Chen et al., 2004).

The dietary taurine requirements in juvenile stages of marine carnivorous fish species such as olive flounder, yellowtail, European seabass and red seabream have range from 0.2 to 2.0% of dry diet (Matsunari et al., 2008). We have recently reported that taurine supplementation to a fish meal diet improves the growth and affects the feeding behavior of parrot fish. Parrot fish, a subtropical marine fish, is carnivorous species and has been regarded as an emerging aquaculture species because of its high economic value, excellent meat quality and strong resistance to diseases. However, no data are available on the dietary requirement or essentiality of taurine for parrot fish which is carnivorous species and has been regarded as an emerging aquaculture species in Korea (Lim and Lee, 2009). Therefore, the study was designed to evaluate the requirement or essentiality of taurine in FM-based diet with graded levels of tauine.

5.2. Materials and methods

5.2.1. Fish rearing

The feeding trial were conducted at the Marine and Environmental Research Institute, Jeju National University, Jeju, South Korea using juvenile parrot fish *Oplegnathus fasciatus*. The fish were from a genetically homogenous stock obtained by a private hatchery, Jeju Island, and adapted to a commercial diet (Suhyupfeed, co., Ltd, GyeongNam, South Korea). Experimental protocols followed the guidelines of the Animal Care and Use Committee of Jeju National University.

5.2.2. Diets and experimental design

Six experimental diets were formulated to be isonitrogenous (46% crude protein) and isocaloric (22MJ/kg diet) and to meet known requirements of nutrients for parrot fish (Lim and Lee, 2009). The experiment was conducted to verify the essentiality or requirement of taurine in FM-based diet for juvenile parrot fish. Six diets were based on white FM (50%) and casein (10%) as the main protein sources. Taurine was supplemented at 0, 0.2, 0.4, 0.8, 1.2, or 1.6% dry diet (designated as T0, T0.2, T0.4, T0.8, T1.2 or T1.6, respectively). The white FM contained 0.34% of taurine and the analyzed concentration of dietary taurine was 0.19% (T0), 0.37% (T0.2), 0.51% (T0.4), 0.87% (T0.8), 1.23% (T1.2) and 1.74% (T1.8) respectively. Supplemental levels of taurine in the experimental diets were determined from the earlier observation with carnivorous fish species (Matsunari et al., 2008). The dietary formulation, proximate composition and taurine content are presented in Table 5-1.

All diets were prepared in the laboratory. Briefly, all the dry ingredients were minced and mixed in a feed mixer (NVM-16, Gyeonggido, South Korea) and then squid liver oil was added and mixed for 5 min. Distilled water was added to mixture (20 - 30 g/100 g of feed weight) and mixed until a pebble-like consistency was achieved. The mixture was then converted to pellets by a meat chopper machine (SMC-12, Kuposlice, Busan, South Korea). Pellets were dried at room temperature for 48 h, crushed into desirable particle sizes and stored in a freezer at –20 ^{o}C until used.

Table 5.1. Formulation and chemical analysis of the diets in Experiment I (% dry matter)

Ingredients	Experimental diets					
	T0	T0.2	T0.4	T0.8	T1.2	T1.6
White fish meal	50.0	50.0	50.0	50.0	50.0	50.0
Casein	11.0	11.0	11.0	11.0	11.0	11.0
Dextrin	23.4	23.4	23.4	23.4	23.4	23.4
Squid liver oil	11.0	11.0	11.0	11.0	11.0	11.0
Mineral mixture[a]	1.0	1.0	1.0	1.0	1.0	1.0
Vitamin mixture[b]	1.0	1.0	1.0	1.0	1.0	1.0
CMC	1.0	1.0	1.0	1.0	1.0	1.0
Cellulose	1.6	1.4	1.2	0.8	0.4	0.0
Taurine[c]	0.0	0.2	0.4	0.8	1.2	1.6
Chemical analysis						
Dry matter, %	9.0	9.3	9.7	9.3	9.2	9.5
Protein, %	47.0	47.1	47.5	47.6	48.0	48.5
Lipid, %	14.9	15.1	14.7	14.9	15.0	15.2
Ash, %	9.1	9.0	9.0	9.2	9.0	9.1
Taurine, %	0.19	0.37	0.51	0.87	1.23	1.74

[a] Mineral premix (g kg^{-1} mixture) $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; CuCl, 0.2; $AlCl_3$. $6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[b] Vitamin premix (g kg^{-1} mixture) L-ascorbic acid, 121.2; DL-α tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-$_D$-pantothenate, 12.7; myo-inositol, 181.8; $_D$-biotin, 0.27; folic acid, 0.68; p-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003.

[c] Taurine, Sigma-Aldrich, St. Louis, Mo, USA

5.2.3. Feeding trials

Twenty fish (IBW, 13.5±0.01 g g/fish) were randomly distributed into each of eighteen 80 L polyvinyl circular tanks (3 replicates per diet). The tanks were in line with a flow through system supplied with sand filtered seawater at a flow rate of 2.5 L/min and oxygenated to above 80% saturation by an air supply. Water temperature was naturally ranged from 20 to 23 ^{o}C, while the photoperiod was maintained at 12 h light/12 h dark during the feeding period. All tanks were cleaned as necessary. Before starting the feeding trial, fish were acclimated for a week to the experimental conditions and fed on the FM-based control diet. Then, the triplicate groups of fish were fed twice per day (8:30 h and 17:30 h) to apparent satiation, 7 days per week, for 8 weeks in accordance with normal parrot fish culture practice. Uneaten feeds were collected 30 min after feeding, dried and reweighed to determine feed intake and feed conversion ratio. Weight gain and feed conversion factor were measured every 2 weeks and feeding was stopped 24 h prior to weighing.

5.2.4. Sample collection and analytical methods

At the beginning and the end of feeding trials, all the fishes were weighed. Weight gain, specific growth rate, feed efficiency, protein efficiency ratio and survival were calculated. Blood samples were obtained from the caudal vein of 6 fishes from each tank (18 fishes per dietary treatment) using heparinized syringe after anesthetization of the fish with tricaine methanesulfonate (MS-222) at a concentration of 100 - 200 mg/L. Hematocrit and hemoglobin were measured using microhematocrit technique (Brown, 1980) and CH 100 plus blood biochemical auto analyzer (SLIM, SEAC Inc, Florence, Italy), respectively. Analyses of crude

protein, moisture and ash in the diets were performed by the standard procedures (AOAC International, 1997) and dietary lipid was determined according to the method described by Folch et al. (1957). Amino acid compositions in the diets were determined using a Sycom S-433D automatic amino acid analyzer (Sykam, Eresing, Germany). Hydrolysis of the flour samples was performed in 6N HCl at 110℃ for 24 h under nitrogen atmosphere. Identification and quantification of amino acids were achieved by comparing the retention times of the peaks with those of standards.

5.2.5. Statistical analysis

All experimental diets were assigned by a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the differences in mean values were made with Duncan's multiple range test. Statistical significance was determined by setting the aggregate type I error at 5% ($P<0.05$) for each set of comparisons. Data are presented as mean±SD. Percentage data were arcsine transformed before statistical analysis. The dietary taurine requirement of juvenile parrot fish was estimated using the broken-line regression method (Robbins, 1986).

5.3. Results

The experimental fish readily accepted all the six experimental diets and maintained normal behavior during the feeding trial. Survival of juvenile parrot fish for all dietary treatments ranged from 95.0% to 100% and there were no significant differences among various dietary treatments (Table 5-2). However, fish growth and feed utilization were positively affected by dietary taurine contents. Weight gain (WG), specific growth rate (SGR), feed efficiency (FE) and protein efficiency ratio (PER) were significantly improved by dietary taurine levels up to 0.87%, and then the responses reached a plateau. Broke-line regression analysis on WG estimated the dietary requirement for taurine to be 0.91% of dry diet for juvenile parrot fish (Fig. 5-1).

At the end of 8 weeks of feeding trial, blood parameters were significantly affected by dietary taurine contents (Table 5-3). Hematocrit value followed the similar trend to the result of WG and groups of fish fed the diets with ≥0.87% taurine had significantly higher hematocrit than that of fish fed the diet with 0.19% taurine. Hemoglobin and plasma cholesterol concentration were also significantly affected by dietary taurine contents. Hemoglobin concentration was significantly increased in fish fed the diets with ≥0.37% taurine compared to that of fish fed the diet with 0.19% taurine.

Table 5-2. Growth performance of juvenile parrot fish in the feeding trials fed different experimental diets for 9 weeks.

Diets	T0	T0.2	T0.4	T0.8	T1.2	T1.6
Dietary taurine, %	0.19	0.37	0.51	0.87	1.23	1.74
WG (%)[a]	215±8.5[a]	222±2.0[a]	229±9.7[ab]	243±6.4[bc]	242±13.5[bc]	249±10.2[c]
SGR (%)[b]	2.08±0.05[a]	2.13±0.01[a]	2.16±0.05[ab]	2.24±0.03[bc]	2.24±0.07[bc]	2.27±0.05[c]
FE[c]	0.61±0.01[a]	0.63±0.01[a]	0.64±0.02[ab]	0.67±0.02[bc]	0.67±0.03[bc]	0.68±0.02[bc]
PER[d]	1.29±0.01[a]	1.32±0.01[ab]	1.36±0.04[b]	1.42±0.03[c]	1.45±0.02[c]	1.43±0.04[c]
Survival (%)	98.3±2.9	95.0±5.0	100±0.0	96.7±2.9	96.7±2.9	95.0±5.0

Means of triplicate groups, values are presented as mean ± SD. Values in the same row having different superscript letters are significantly different ($P < 0.05$).

[a] Weight gain (%) = 100 x (final mean body weight - initial mean body weight) x initial mean body weight^{-1}

[b] Specific growth rate (%) = [(loge final body weight - loge initial body weight) x days^{-1}] x 100

[c] Feed efficiency = wet weight gain x dry feed fed^{-1}

[d] Protein efficiency ratio = wet weight gain x total protein given^{-1}

Table 5-3. Blood parameters of juvenile parrot fish in the feeding trials fed different experimental diets for 9 weeks.

Diets	T0	T0.2	T0.4	T0.8	T1.2	T1.6
Dietary taurine, %	0.19	0.37	0.51	0.87	1.23	1.74
Hematocrit (%)	39.8±1.3^{a}	42.6±1.7^{ab}	42.6±2.5^{ab}	45.3±1.3^{b}	43.8±1.3^{b}	43.5±0.5^{b}
Hemoglobin (g/dL)	7.0±0.2^{a}	7.6±0.2^{b}	7.8±0.3^{b}	8.1±0.2^{b}	7.8±0.1^{b}	7.7±0.4^{b}

Mean values of triplicate groups, values are presented as mean ± SD. Values in the same row having different superscript letters are significantly different ($P < 0.05$).

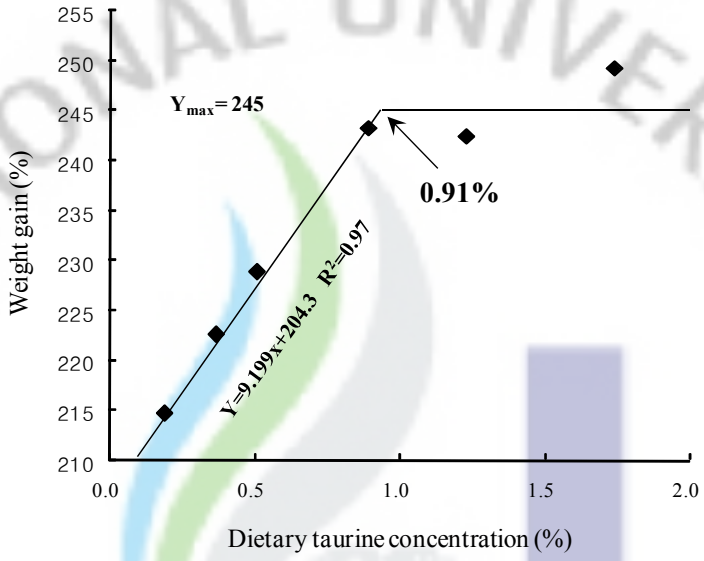

Fig. 5-1. Broken-line analysis of weight gain on concentrations of dietary taurine indicates that the optimal taurine level for maximal growth of juvenile parrot fish is 0.91% dietary taurine. Each point represents the mean of three groups of fish (n=3).

5.4. Discussion

In this study, we provide evidence that supplementation of taurine to the fish meal based diet significantly improves the growth performance of juvenile parrot fish. Recent studies have demonstrated that several fish species require dietary taurine as an essential nutrient (Park et al., 2001; Matsunari et al., 2005, 2008) since the ability of these fish species to synthesize taurine is markedly low (Goto et al., 2001; Yokoyama et al., 2001). In general, taurine is synthesized through sulfur amino acid metabolism in mammals (Yamaguchi, 1985). However, kitten cannot synthesize taurine adequately, and requires this amino acid as an essential nutrient (Knopf et al., 1978). Among teleosts, rainbow trout synthesizes taurine via this pathway (Goto et al., 2001; Yokoyama et al., 2001). This has been demonstrated by the result that tissue taurine concentration of rainbow trout is increased by dietary methionine and cystine supplementation (Walton et al., 1982; Cowey et al., 1992; Yokoyama and Nakazoe, 1992). Although taurine is one of the most abundant free amino acids in fish tissue, it is not incorporated into protein (Hujita, 1988). Hence, taurine has not been considered as an essential nutrient for fish to date (Borlongan and Coloso,1993; Akiyama et al.,1997; Forster and Ogata, 1998). However, recent studies have indicated that taurine synthesis widely differs among fish species, and the rate of synthesis in species such as bluefin tuna, yellowtail, olive flounder and red sea bream is low or negligible (Goto et al., 2001; Yokoyama et al., 2001). Furthermore, it has been reported that taurine requirements of juvenile yellowtail (0.5 g in initial body weight) (Matsunari et al., 2005) and juvenile Japanese flounder (0.15 g in initial body weight) (Park et al., 2001) fed FM based diets are more than 10 g/kg diet and 15–20 g/kg diet, respectively, but the requirements decrease with increase in the fish size. On the other hand, the previous studies (Takagi et al., 2006) indicate that yellowtail (250–450 g in initial body weight) fed a non-FM diet based on soy protein concentrate as the protein source require 45 g taurine/kg of diet. The quantitative

requirement of taurine in yellowtail fed a soy protein concentrate diet (Takagi et al., 2006) is considerably higher than that of fish fed a FM based diet (Matsunari et al., 2005), although body weight of the fish examined by Takagi et al. (2006) was considerably larger than the fish studied by Matsunari et al. (2005).

Broke-line regression analysis on WG against the dietary taurine levels indicates that the optimum level of taurine to the fish meal based diet (50%) is about 0.9% in juvenile parrot fish. This value is lower than those for other fish species such as olive flounder (1.5-2.0%) and yellowtail (>1.0%) and is higher than European seabass (requirement, 0.2%) and red seabream (0.5%). Such variations in taurine requirements among the fish species may partly be a result of the differences in fish size examined. In olive flounder, the efficacy of taurine supplementation for improving growth performance has been observed only during the juvenile period (0.4 g) but not during the fingerling period (15 g) (Kim et al., 2003). On the other hand, Takagi et al. (2006) reported that only 0.2% supplemention to the low fish meal diet with soy protein concentrate improved the growth performance of yearling red seabream. Soybean meal is known to contain several antinutritional components such as lectins, saponins, phytoestrogens and allergens (Francis et al., 2001). It was reported that cats, which require taurine, fed soybean protein diet had significantly lower plasma taurine levels than cats fed a casein diet. In addition, the type and quantity of the protein in the diet affects the taurine status in cats (Kim et al., 1995). Thus, the taurine requirement of red seabream may depend on protein sources and their inclusion levels in diets. As such, additional research is needed to determine the taurine requirement of parrot fish at different growth stages as well as diets containing different proteins.

It is known that hematocrit and hemoglobin values are usually used as indicators of general health in fish (Tort et al., 1996). Many researchers have found that hematocrits and hemoglobin

varies according to the deficiency of essential nutrients, environmental conditions, growth status or anti-nutritional factors (Garrido et al., 1990; Lim and Lee, 2009). In this study, therefore, the reason for the lower blood hematocrit and hemoglobin in fish fed the diet without supplemental taurine might be mainly due to the insufficiency of dietary taurine.

In conclusion, the optimal dietary taurine requirement for juvenile parrot fish (13 - 45g) fed fish meal based diet (50% white fish meal) was estimated to be 0.9%. The results of the present study suggest that the supplementation of taurine to a fish meal or plant based diet promotes the feed intake and growth performance in practical diets for juvenile parrot fish.

SUMMARY

Dietary replacement of fish meal has been an important issue in aquaculture industry due to a limited supply of FM and its dramatic price increase in recent years. Feed costs account for over 50% of total production costs in most marine fish species, because of the use of the expensive FM with a large dietary proportion. Plant origin byproducts have been promising candidates for the FM replacement and successfully used in many fish species. However, FM cannot be totally replaced by plant protein sources without significant reduction in fish performance or feed utilization for most species investigated. Even when experimental diets meet the known requirements of fish, they still generally perform poorer growth performances and feed utilization than high fish meal diets partly. The reduced growth performance by fish fed plant protein sources is partially explained by the presence of anti-nutritional factors. However other differences between protein sources of plant and marine origin could also be of importance. For instance, taurine has been shown to improve growth in several carnivorous fish species. Recent studies have demonstrated that several fish species require dietary taurine as an essential nutrient. This phenomenon could be explained by their feeds in nature that contain high level of taurine. In wild environment, therefore, the fish species can be satisfied with required taurine for growth or normal physiological functions by their feeds, thus may be less able to synthesize taurine while herbivorous fish may be more capable of taurine biosynthesis because of lack of taurine in their feeds. The current study was undertaken to confirm necessity of taurine supplementation for maximum growth of juvenile parrot fish fed a fish meal or plant based diets and to determine whether the taurine supplementation to plant protein based diet would improve growth performance of juvenile parrot fish.

The results indicated that plant protein sources, such as cottonseed and soybean meal with

iron and phytase supplements could replace dietary FM protein up to 20 ~30% without negative effects on growth performances, feed utilizations and survival of juvenile or growing parrot fish (Chapter two). The dietary supplementation of taurine to a soybean meal based diet promotes the feed intake and growth performance and 30% fish meal were able to be replaced by soybean meal with supplementation of 1% taurine in the presence of lysine, methionine and phosphorus for juvenile parrot fish (Chapter three). The dietary taurine supplementation does have a significant impact on growth and feed efficiency of growing parrot fish when they are fed diets containing high levels of plant protein sources as replacements for fish meal. Soybean meal with taurine supplementation could replace dietary FM protein up to 40% in growing parrot fish (Chapter four). Taurine is an essential amino acid for juvenile parrot fish and the optimal dietary taurine requirement for the fish on fish meal based diet (50% white fish meal) was estimated to be 0.9% (Chapter five).

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LIST OF PUBLICATIONS

International Journals

1. Pham, M.A., Lee, K.J., Lee, B.J., Lim, S.J., Kim, S.S., Lee, Y.D., Heo, M.S., Lee, K.W. (2006) Effects of dietary *Hizikia fusiformis* on growth and lmmune responses in juvenile olive flounder (*Paralichthys plivaceus*). *Asian-Aust. J. Anim. Sci* 19, 1769-1775.
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